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Introduction

1 La biologie des systèmes

La biologie des systèmes cherche à intégrer les différents niveaux d'information qui structurent un organisme. Ces niveaux d'information s'établissent à différentes échelles : macroscopique comme les mécanismes physiologiques, microscopique comme la communication cellulaire ou nanométrique comme la régulation génique. Interconnecter ces différents niveaux permettra, à terme, de comprendre, de prédire, voire de contrôler la dynamique de fonctionnement global d'un système vivant. La difficulté d'une telle approche vient du fait que chaque niveau est régi par des lois qui lui sont propre. Ainsi, avant de pouvoir construire un modèle global multi-échelle d'un système vivant complexe, il est nécessaire de diviser les difficultés en se focalisant à un niveau d'information donné sur un organisme suffisamment simple. Dans cette étude, nous nous focaliserons sur la dynamique de fonctionnement du réseau de régulation d'un organisme très étudié et facilement manipulable : la bactérie *Escherichia coli*.

L'adaptation d'une bactérie aux changements dans son environnement est contrôlée au niveau moléculaire par un réseau de régulation large et complexe impliquant des gènes, des ARN, des protéines et des métabolites. Transduction du signal, régulations transcriptionnelles, flux métaboliques sont étroitement interconnectés même si ils fonctionnent à des échelles de temps très différentes (de la milliseconde au jour). Connaître la topologie de ce réseau de régulation est le premier objectif de la biologie des systèmes. La base de données Ecocyc liste plus de 5345 interactions régulatrices chez *E. coli* et ce nombre ne cesse d'augmenter (Keseler *et al.*, 2010). Etudier le fonctionnement dynamique de ce réseau est le second objectif de la

biologie des systèmes. Cette dynamique est non linéaire et comprend de nombreuses boucles de rétroaction. De plus, la nature intrinsèquement stochastique des mécanismes de régulation (mouvement brownien) rend le réseau de régulation idiosyncratique : pour un même génotype, une topologie du réseau identique et une même perturbation, deux bactéries peuvent adopter deux phénotypes distincts lors de l'adaptation (Veening *et al.*, 2008).

En l'état actuel des connaissances, il n'est pas encore possible de comprendre et de contrôler finement la dynamique de fonctionnement de cet énorme réseau de régulation. Nous nous sommes donc focalisés sur un module de régulation plus restreint mais faisant intervenir à la fois une réorganisation de la transcription et une réorganisation des flux métaboliques : nous avons choisi, comme point de départ, d'étudier l'adaptation de la bactérie *E. coli* à un changement de sources de carbone : du glucose à l'acétate (Wolfe, 2005).

2 Le système d'étude

Lorsque les bactéries *E. coli* sont placées dans un milieu contenant du glucose, elles consomment ce glucose tout en secrétant de l'acétate dans le milieu extérieur. Une fois le glucose épuisé, les bactéries réimportent cet acétate et l'utilisent comme source d'énergie. Cette transition glucose-acétate entraîne une inversion des flux métaboliques (de la glycolyse à la néoglucogenèse) et une profonde réorganisation de l'expression des gènes (Oh *et al.*, 2002).

Pour pouvoir utiliser l'acétate comme source d'énergie principale, il est nécessaire de le convertir en acétyl-coenzyme A. Cette première étape est réalisée par l'acétyl-coenzyme A synthétase encodée par le gène *acs* (Kumari *et al.*, 1995). Ce gène a un rôle précoce et prédominant dans l'établissement de la transition glucose-acétate c'est pourquoi nous nous sommes focalisés sur la régulation de son expression. En effet, sa transcription est connue pour être activée par le facteur de transcription Crp qui n'est actif que lorsque le glucose est épuisé (Kumari *et al.*, 2000). Ce mécanisme, appelé répression catabolique, fait intervenir une petite molécule : l'adénosine monophosphate cyclique (AMPc). Selon un modèle classique, lorsque le glucose est épuisé, une cascade de réactions aboutit à l'activation d'une enzyme, l'adenylate cyclase, qui produit alors une forte concentration d'AMPc. Cette molécule se fixe à la protéine Crp et change sa conformation. Le complexe ainsi formé a une plus grande affinité pour son site de liaison à l'ADN, au niveau de la séquence promotrice du gène *acs*. La fixation de ce complexe Crp-AMPc entraine l'activation de la transcription de ce gène.

Pour pouvoir mesurer l'expression du gène *acs* avec une haute résolution temporelle, nous utilisons la technologie des fusions transcriptionnelles. Nous fusionnons, sur un plasmide multi-

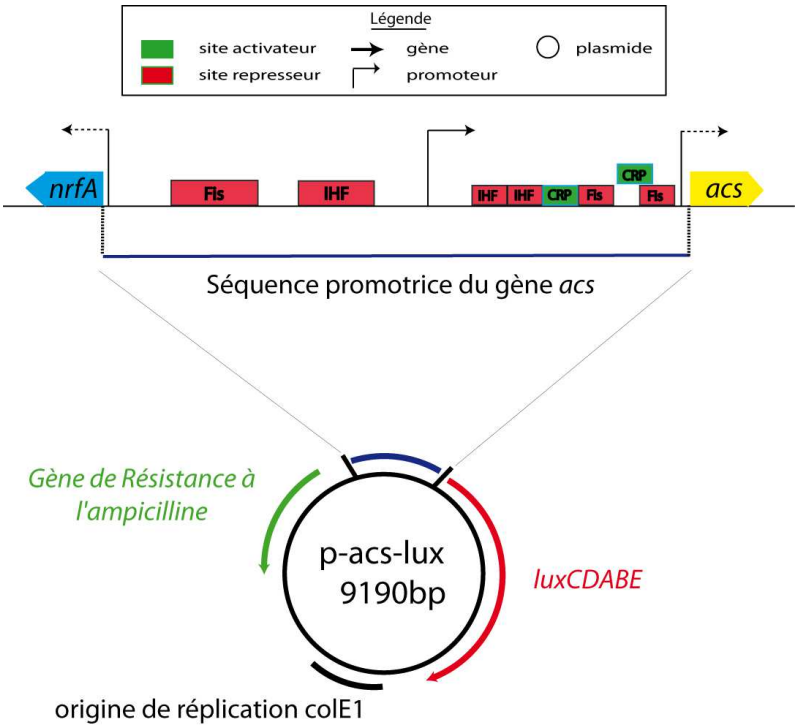

Figure I.1 – Fabrication de la fusion transcriptionnelle p-acs-lux. La première étape consiste à amplifier la région promotrice du gène *acs* à partir du chromosome bactérien. Puis cette région est clonée en amont du gène rapporteur (*luxCDABE*) sur un plasmide multicopie. Cet « instrument » permet d'inférer l'activité du promoteur *acs* à partir du signal de luminescence généré.

copie, la séquence promotrice du gène *acs* en amont d'un gène rapporteur (l'operon *luxCDABE*) codant pour la luciferase : cet outil serait désormais appelé p-acs-lux (Figure I.1). Ainsi l'activité du promoteur *acs* est directement reliée à la concentration de luciferase qui est, elle-même, proportionnelle à la production de lumière, facilement mesurable Une fois notre outil construit, nous l'avons placé, par transformation, dans des bactéries *E. coli* sauvages et nous avons fait pousser ces dernières dans un milieu minimum contenant du glucose et de l'acétate. La croissance a lieu dans un lecteur de microplaque ce qui nous permet de mesurer, à intervalle de temps régulier, l'absorbance qui reflète le nombre de bactéries et la luminescence qui reflète l'activité du promoteur *acs*. Avec cette méthode, nous avons pu observer que l'activité du promoteur *acs* est fortement réprimée durant la croissance sur glucose, puis augmente brutalement lorsque ce dernier est épuisé (Figure I.2). Pour s'assurer que ce profil d'expression est correct, nous l'avons contrôlé à l'aide d'une autre fusion transcriptionnelle composée d'un autre rapporteur : la *gfp*. Le profil obtenu en fluorescence est similaire à celui obtenu en luminescence. Enfin, pour définitivement exclure tout artefact, nous avons également confirmé ce profil à l'aide d'une méthode totalement indépendante : la qRT-PCR.

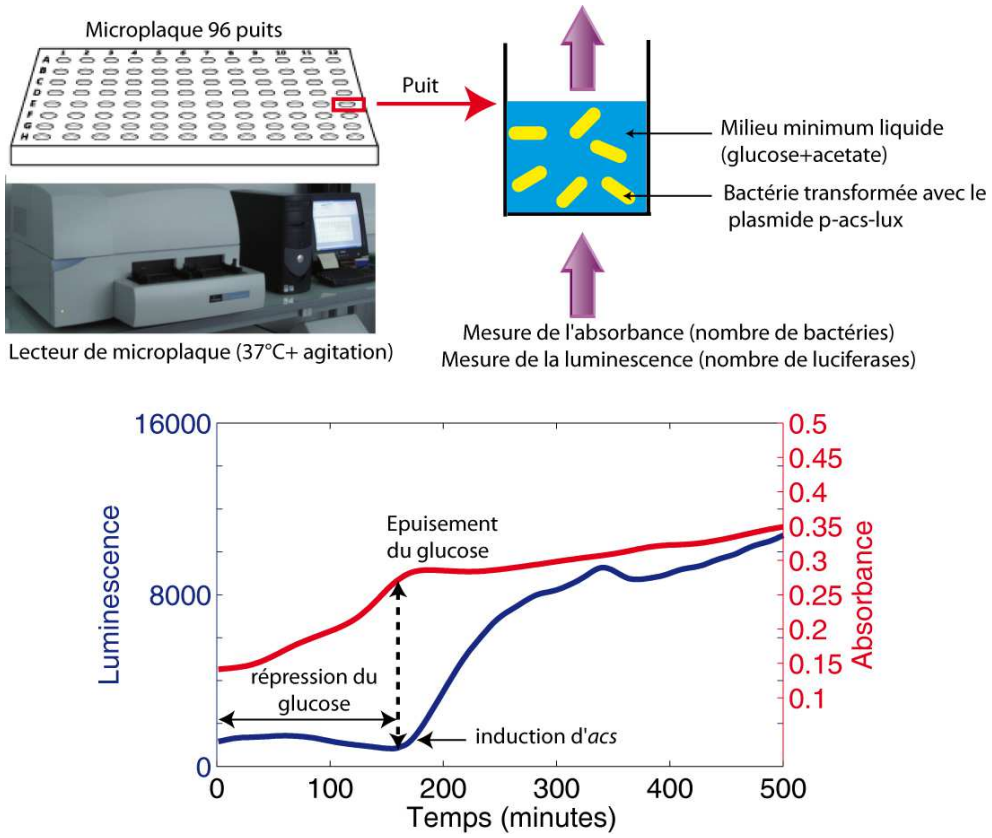

Figure I.2 – Mesure de l'expression du gène *acs* dans une souche sauvage. Les mesures d'expression génique sont effectuées dans une microplaque incubées à 37°C sous agitation. Des bactéries sauvages, contenant le plasmide p-acs-lux, sont placées dans un milieu minimum liquide avec du glucose et de l'acétate. Le lecteur de microplaque mesure l'absorbance et la luminescence toutes les 5 minutes pendant plusieurs heures. La courbe rouge correspond à la courbe de croissance. On remarque un point de cassure qui signale l'épuisement du glucose. C'est à ce moment là qu'a lieu une forte induction de luminescence correspondant à une forte expression du gène *acs* (courbe bleue).

3 Logique de régulation de l'expression d'*acs*

Dans les paragraphes qui suivent, nous allons nous intéresser à la cascade d'événements qui a lieu entre l'épuisement du glucose et l'activation du promoteur *acs*. C'est en effet cette cascade d'événements qui est en mesure d'expliquer l'allure du profil d'expression décrit précédemment. La littérature décrit l'activation de l'expression du gène *acs* par le facteur de transcription Crp. Nous avons vérifié cette information en mesurant son expression dans un mutant Δcrp. Comme prévu, dans une telle souche, il n'y a aucune expression du gène *acs*. Ceci entraîne un phénotype facile à observer : l'absence de croissance sur acétate. En réintroduisant, dans ce mutant, une copie du gène *crp*, on retrouve l'expression du gène *acs* et la croissance sur acétate. Les choses se passent de manière très similaire dans un mutant $\Delta cyaA$, c'est à dire

dans un mutant sans adenylate cyclase. En absence d'AMPc, le facteur de transcription Crp ne peut pas se lier à la séquence promotrice du gène *acs* et ce dernier n'est pas exprimé. A l'inverse, l'ajout d'AMPc exogène dans le milieu rétablit l'expression du gène *acs*. Le résultat de cette dernière expérience a mis en évidence une divergence vis-à-vis du modèle classique de la répression catabolique. L'ajout d'AMPc dans le milieu aurait dû contourner la répression du glucose et induire directement l'expression du gène *acs* malgré la présence de glucose. Or ce n'est pas ce que nous observons : l'expression d'*acs* dans le mutant $\Delta cyaA$ est complémentée par l'ajout d'AMPc exogène, mais seulement après épuisement du glucose (Figure I.3). Nous avons donc déduit qu'il existait un autre mécanisme régulateur empêchant l'expression d'*acs* tant qu'il y a du glucose dans le milieu.

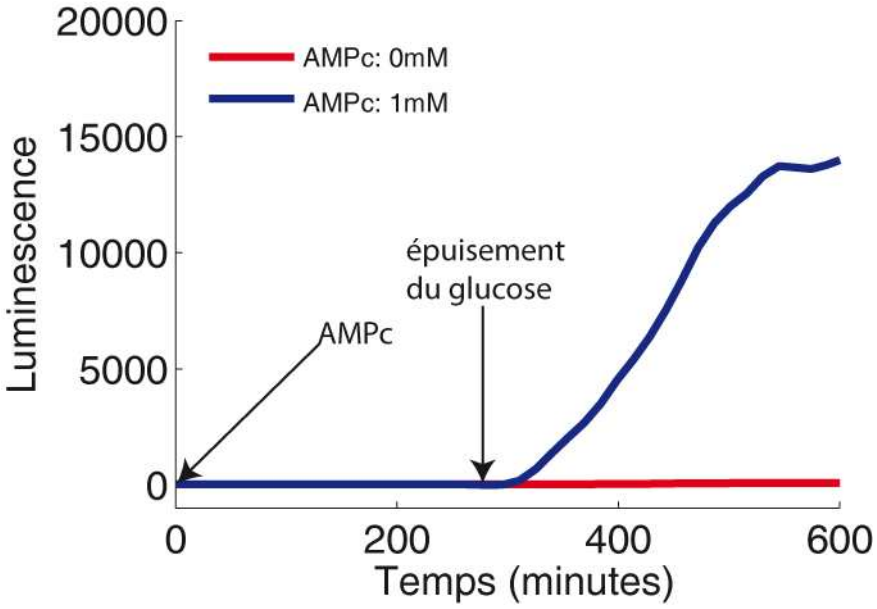

Figure I.3 – Expérience phare montrant une incohérence dans le modèle de la répression catabolique. nous mesurons l'expression du gène *acs* dans une souche $\Delta cyaA$ poussant dans un milieu liquide avec du glucose et de l'acétate. Sans AMPc (courbe rouge), il n'y a aucune expression du gène *acs*. La présence d'AMPc exogène depuis le début de la cinétique permet de rétablir l'expression d'*acs*. Pourtant ce rétablissement a lieu seulement une fois que le glucose est épuisé.

4 Un criblage à haut-débit pour détecter de nouvelles interactions géniques

Pour rechercher ce mécanisme de régulation inconnu, nous avons développé un nouveau type de criblage à haut-débit. Nous avons transformé les 4000 simples mutants d'*Escherichia coli*,

provenant d'une collection (Baba et al. 2006), avec notre fusion transcriptionnelle p-acs-lux. Le criblage consiste à comparer la luminescence des colonies mutantes avec celle de la colonie sauvage : une différence significative pouvant suggérer que le gène déleté contrôle, directement ou indirectement, l'expression du gène *acs* (Figure I.4). Parmi les 4000 mutants, nous avons identifié deux groupes de gènes bien distincts. Le premier groupe est composé de gènes impliqués dans la fabrication de l'énergie. La luciferase, pour émettre des photons, a besoin de pouvoir réducteur ce qui implique la présence d'un métabolisme central fonctionnel (Meighen, 1991). Certaines colonies mutantes, émettant significativement moins de lumière, révèlent un épuisement énergétique plutôt qu'une régulation spécifique sur l'activité du promoteur *acs* : il s'agit donc de faux positifs. Nous reviendrons plus tard en détail sur cet artefact énergétique pour montrer qu'il peut également être utile.

Un deuxième groupe de gènes a été identifié par notre criblage : les gènes du système de transfert de phosphate (PTS). Les enzymes correspondantes sont impliquées dans l'import et la phosphorylation du glucose pour donner du glucose-6-phosphate. Le phosphate ajouté a subi une cascade de transfert partant de phospho-enol-pyruvate (PEP) et se déplaçant successivement sur l'enzyme E1 (*ptsI*), puis HPR (*ptsH*) puis EIIA_{glc} (*crr*), puis sur le transporteur EIIB/C_{glc} (*ptsG*) pour se retrouver enfin sur le $6^{ème}$ carbone du glucose formant le glucose-6-phosphate (Postma et al. 1993).

La détection par notre criblage des gènes appartenant au PTS n'est pas étonnante en soi. En effet, l'implication de ce système dans la répression catabolique est connue depuis longtemps. Ce système fait le lien entre l'épuisement du glucose et l'augmentation de la concentration d'AMPc dans la cellule. Le modèle actuel postule que seule la forme phosphorylée de l'enzyme EIIA_{glc} soit capable d'activer l'adenylate cyclase et donc d'augmenter la synthèse d'AMPc. Or la forme phosphorylée d'EIIA_{glc} est la version prédominante en absence de glucose (schéma récapitulatif de la figure I.5). Notre criblage n'a pas seulement réussi à faire ressortir les mutants du PTS, il a également révélé de nouvelles subtilités dans le fonctionnement de la répression catabolique. En effet, les colonies Δcrr (sans EIIA_{glc}) et $\Delta ptsI$ (sans E1) ont été sélectionnées pour leur faible luminescence alors que la colonie $\Delta ptsG$ (sans EIIB/C_{glc}) a été sélectionnée sur la base de sa forte luminescence. Ce résultat n'est ni prédictible ni explicable par le modèle ci-dessus.

Un des avantages de notre méthode à haut-débit est d'offrir la possibilité de mener des investigations plus poussées en testant les phénotypes intéressants dans différentes conditions et en dynamique. Par exemple, nous avons pu vérifier que, dans mutant Δcrr (sans EIIA_{glc}), l'ajout d'AMPc exogène dans le milieu rétablit l'expression du gène *acs*. Ce résultat suggère (sans le démontrer) le mécanisme d'activation de l'adenylate cyclase par la version phosphorylée de EIIA_{glc}. Pourtant (et comme observé avec le mutant $\Delta cyaA$) dans le mutant Δcrr, l'AMPc

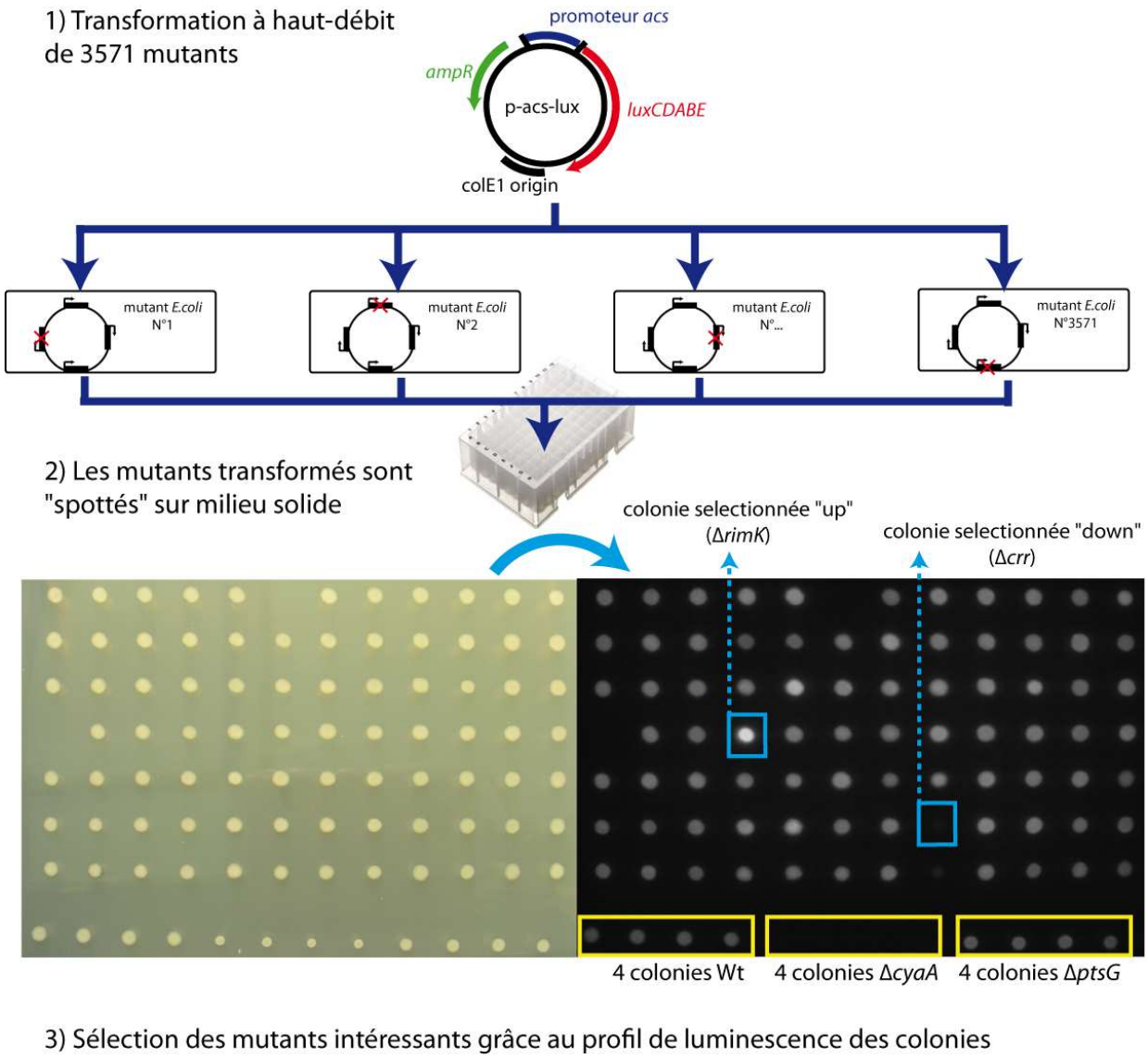

Figure I.4 – Un nouveau criblage à haut-débit pour détecter de nouvelles interactions géniques. Nous transformons la fusion transcriptionnelle p-acs-lux dans l'ensemble des simples mutants d'*E. coli*. Puis nous « spottons » sur boite de petri (il est possible de tester différents types de milieu) les transformants. Après une nuit à 37°C, les colonies apparaissent. Le profil de luminescence de ces colonies est mesuré à l'aide d'une caméra sensible. Les colonies très luminescentes (par rapport à la souche sauvage) peuvent révéler l'absence d'un répresseur transcriptionnelle. A l'inverse, les colonies peu luminescentes peuvent révéler l'absence d'un activateur.

rétablit l'activité du promoteur *acs* seulement une fois que le glucose est épuisé : il subsiste donc un mécanisme de répression indépendant de l'enzyme $EIIA_{glc}$. Nous allons voir que ce résultat d'expérience contredit un deuxième modèle de la répression catabolique.

5 Recherche du mécanisme répresseur

Il existe une deuxième explication au phénomène de répression catabolique : le mécanisme d'exclusion de l'inducteur (Saier & Roseman, 1972). Beaucoup de gènes chez *E. coli* ont leur expression contrôlée par un couple facteur de transcription-inducteur. Citons le couple classique LacI-lactose[1]. Dans le modèle de l'exclusion de l'inducteur, c'est, cette fois, la forme déphosphorylée de $EIIA_{glc}$ qui inhibe l'import du lactose au niveau du transporteur. Or la forme déphosphorylée d'$EIIA_{glc}$ est prédominante en présence de glucose (schéma récapitulatif de la figure I.5). L'inhibition de l'import du lactose empêche ce dernier de jouer son rôle d'inducteur.

Dans le cas d'*acs*, il n'existe pas de facteur de transcription connu qui, en présence d'acétate, activerait ou de-réprimerait l'expression du gène *acs*. Notre criblage n'a d'ailleurs pas détecté l'existence d'un tel facteur. Il est difficile d'exclure expérimentalement l'hypothèse du rôle « inducteur » de l'acétate car ce dernier est produit et sécrété par la bactérie elle-même. Cependant, si la version déphosphorylée de $EIIA_{glc}$ inhibait l'entrée de l'inducteur potentiel « acétate », alors l'ajout d'AMPc exogène dans le milieu de croissance d'un mutant Δcrr (sans $EIIA_{glc}$) devrait contourner la répression exercée par le glucose via $EIIA_{glc}$. Or ce n'est pas ce que nous observons. Ainsi ni le modèle d'exclusion de l'inducteur ni le modèle passant par l'activation de l'adenylate cyclase ne sont en mesure d'expliquer nos données.

A partir de là, il restait une dernière hypothèse à exclure : l'AMPc exogène que nous ajoutons pourrait ne pas réussir à rentrer dans la bactérie en présence de glucose ce qui pourrait expliquer l'absence de levée de répression. D'autant que la bactérie *E. coli* secrète des quantités non négligeables d'AMPc dans le milieu extérieur sans que l'on connaisse la fonction de cet AMPc secrété. Ce pool extérieur d'AMPc pourrait être la résultante d'un mécanisme d'import/export servant à contrôler finement la concentration d'AMPc intracellulaire. Pour exclure cette hypothèse, nous avons utilisé une protéine dite Crp*, qui est une copie de Crp légèrement modifiée (Khankal *et al.*, 2009). Cette protéine a la particularité d'être « AMPc indépendante » : elle peut activer les promoteurs Crp-AMPc dépendants sans nécessité la formation du complexe Crp-AMPc. Nous avons donc construit une souche $\Delta crr\ \Delta cyaA\ crp^*$. Quand cette souche pousse dans un milieu avec du glucose et de l'acétate (et sans AMPc exo-

[1] En réalité l'allolactose

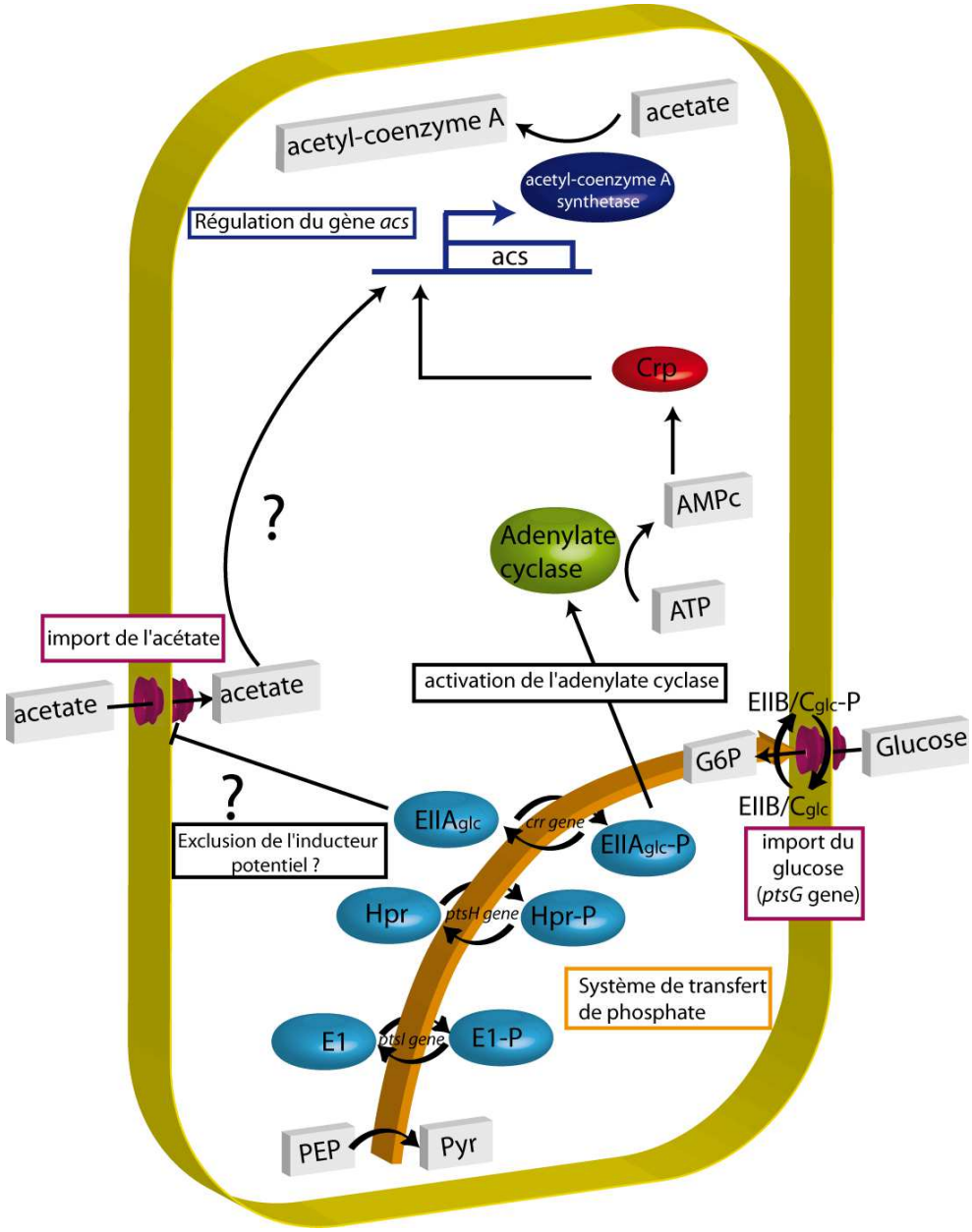

Figure I.5 – Schéma récapitulatif des deux modèles principaux de la répression catabolique. Dans le modèle « AMPc », l'arrêt de l'import de glucose, entraine la phosphorylation des enzymes du système de transfert de phosphate. L'enzyme $EIIA_{glc}$, dans sa version phosphorylée, active l'adenylate cyclase ce qui augmente la concentration d'AMPc. Cette molécule se fixe ensuite au facteur de transcription Crp qui active alors l'expression du gène *acs*. Bien que le rôle de l'acétate comme inducteur du gène *acs* n'ait jamais été montré, il est possible d'imaginer un deuxième mécanisme de répression catabolique : l'exclusion de l'inducteur. Dans ce modèle, durant la croissance sur glucose, l'enzyme $EIIA_{glc}$ est principalement sous forme déphosophorylée. Or, cette forme pourrait inhiber le système d'import de l'acétate et empêcher son rôle hypothétique d'inducteur. La suite de notre exposé montrera que ni l'un ni l'autre de ces modèles ne peuvent expliquer la répression catabolique que nous observons.

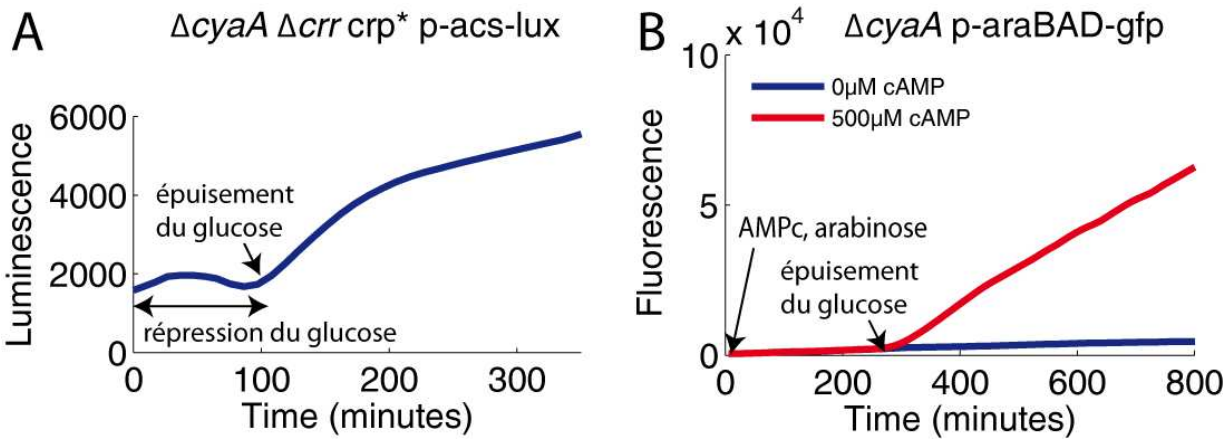

Figure I.6 – Souche *crp** et autres promoteurs Crp-AMPc dépendants. A. Expression du gène *acs* dans une souche Δ*crr* Δ*cyaA* *crp** poussant dans un milieu minimum avec du glucose et de l'acétate. Comme la courbe de croissance n'est pas montrée, nous indiquons l'épuisement du glucose par une flèche. On constate que le glucose réprime l'expression du gène *acs* malgré l'absence d'AMPc et de l'enzyme $EIIA_{glc}$. B. Expression de l'operon *araBAD* dans une souche Δ*cyaA* poussant dans un milieu minimum avec du glucose, de l'acétate, de l'arabinose (l'inducteur) avec ou sans AMPc. Ici le gène rapporteur est la *gfp*, c'est pourquoi nous mesurons la fluorescence et non la luminescence. On constate que l'AMPc ajouté permet bien de rétablir l'expression de l'operon *araBAD* mais seulement une fois que le glucose est épuisé.

gène), le profil d'expression du gène *acs* est très similaire à celui de la souche sauvage : la répression du glucose est toujours visible malgré le fait que les deux pierres angulaires des modèles de la répression catabolique, $EIIA_{glc}$ et l'adenylate cyclase, ont été retirées (Figure I.6A). L'hypothèse du contrôle de l'import/export de l'AMPc, comme explication de nos résultats, devient caduque car la répression du glucose perdure dans cette souche en absence d'AMPc.

6 Un phénomène général

Ces résultats obtenus sont-ils spécifiques à la régulation du gène *acs* ou sont-ils généralisables à l'ensemble des promoteurs Crp-AMPc dépendants ? Pour répondre à cette question, nous avons sélectionné plusieurs fusions transcriptionnelles Crp-AMPc dependantes dans une collection (Zaslaver *et al.*, 2006). Il s'agit des promoteurs *araBAD*, *glpA* et *sdhC*. Bien qu'il existe des subtilités quantitatives, nous avons pu montrer que les résultats obtenues avec *acs* sont généralisables à ces 3 promoteurs (Figure I.6B). Nous pensons donc que nos recherches portent sur le mécanisme général de la répression catabolique.

7 Le flux glycolytique

Dans toutes nos expériences, le paramètre qui semble crucial pour contrôler l'état de la répression catabolique (et donc l'état d'activation du régulon Crp) est l'état du flux glycolytique. En effet, dans le mutant Δ*ptsG* (sans le transporteur du glucose), le niveau basal d'expression d'*acs* est surélevé par rapport à la souche sauvage car le flux glycolytique y est, à l'inverse, réduit (Figure I.7A). Une autre manière pour diminuer le flux glycolytique consiste à utiliser un sucre non métabolisable (l'α-methyl-glucose) agissant en compétition avec le glucose. La littérature décrit ces sucres comme des répresseurs cataboliques alors que nous ne pensons pas qu'ils le soient. Au contraire, lorsque le ratio glucose/α-methyl-glucose génère la réduction adéquate du flux glycolytique, nous avons pu activer l'expression de plusieurs promoteurs Crp-AMPc dépendants (Figure I.7B).

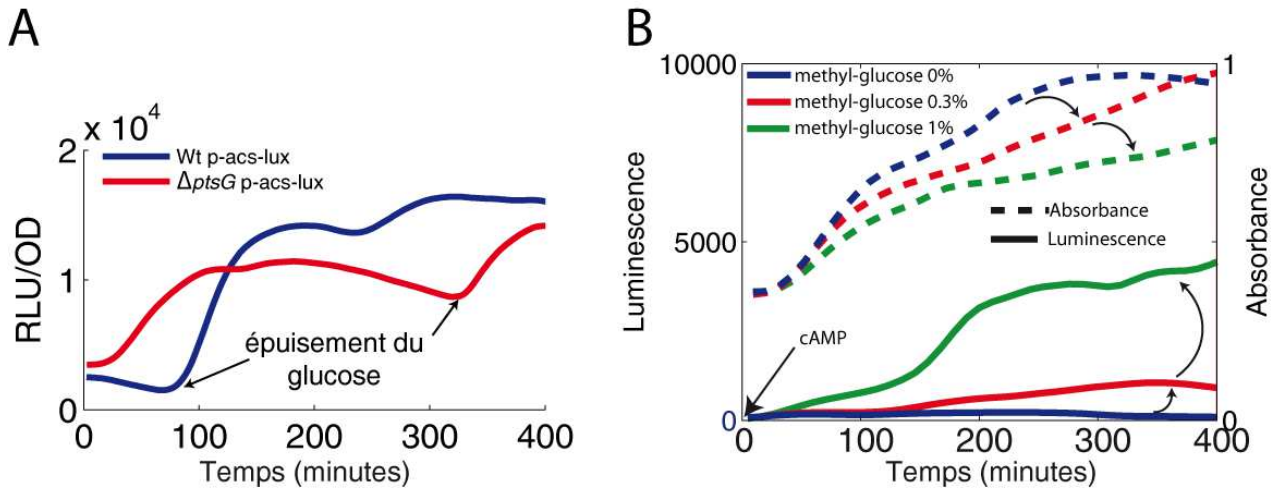

Figure I.7 – Rôle du flux glycolytique. A. Dans un mutant Δ*ptsG* (courbe rouge), déleté du transporteur principal du glucose, poussant sur un milieu minimum avec du glucose et de l'acétate, la consommation du glucose (et donc le flux glycolytique) est plus lente ce qui, d'une part, retarde l'induction d'*acs* et, d'autre part, rehausse son expression par rapport à une souche sauvage. B. Dans une souche Δ*cyaA* rapportant l'expression du promoteur *sdh* (Crp-AMPc dépendant) et poussant sur un milieu avec du glucose et de l'AMPc, il est possible de rehausser l'expression du gène *sdh* de manière dose-dépendante en diminuant la consommation du glucose (ce qui diminue le taux de croissance) à l'aide du methyl-glucose, non métabolisable.

8 Expériences à l'échelle de la cellule individuelle

Les données présentées jusqu'à maintenant sont principalement issues d'expériences effectuées à l'échelle de la population dans les puits d'une microplaque. Nous avons également vérifié

certains de nos résultats à l'échelle de la cellule isolée. Par exemple, nous avons pu mettre en évidence l'induction du gène *acs* lorsque le glucose est épuisé (Figure I.8). De même, nous avons pu observer, sous le microscope, la répression catabolique exercée sur l'expression de l'operon *araBAD* malgré la présence de l'inducteur (arabinose) et de l'AMPc. L'expression de l'operon *araBAD* est connue pour amplifier les effets du bruit moléculaire à cause de la présence d'une boucle de rétroaction positive (Megerle *et al.*, 2008). Nous avons également observé ces variations stochastiques. De plus, nous pensons avoir été en mesure de contrôler finement la distribution de l'expression de cet opéron dans la population de cellules en faisant varier le flux glycolytique à l'aide de l'α-methyl-glucose.

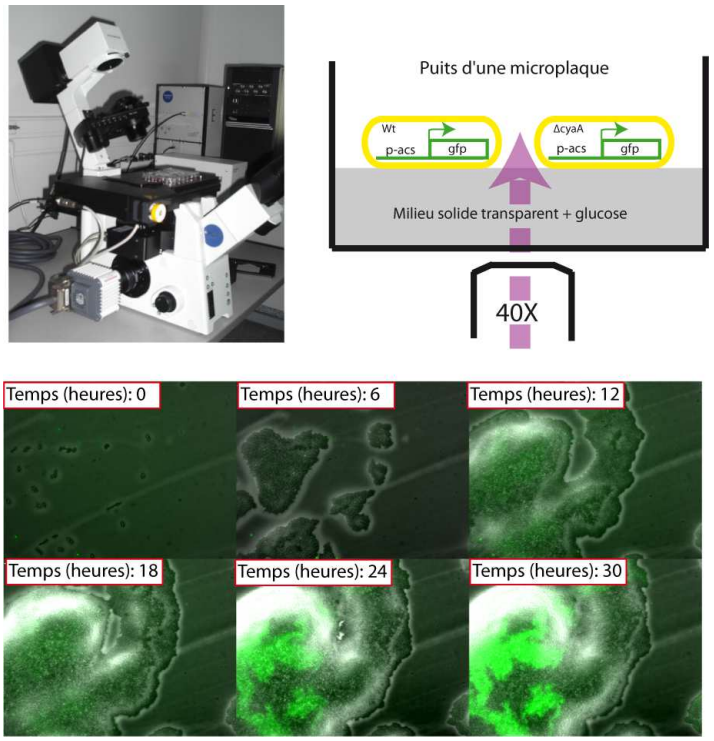

Figure I.8 – Mesure de l'expression du gène *acs* à l'échelle de la microcolonie. Un puits d'une microplaque 6 puits contient un milieu minimum solide (agarose) avec du glucose. Deux types de bactéries sont présentes sur ce milieu : la souche sauvage et la souche $\Delta cyaA$, toutes deux transformées avec un plasmide rapportant l'expression du gène *acs* via la *gfp*. Les deux types de bactéries commencent à former des micro-colonies et ces dernières rentrent en contact et se mélangent. Une fois le glucose épuisé (arrêt de la croissance de la microcolonie), seul le tapis bactérien composé des bactéries sauvages voit sa fluorescence augmenter brusquement, signe de l'expression du gène *acs*.

Notre système expérimental sous le microscope (milieu solide, formation de microcolonies) est très différent de celui en milieu liquide. Pourtant, les résultats obtenues dans ces deux types de conditions sont similaires ce qui démontrent leur certaine robustesse. Cela peut sembler anodin mais le mécanisme responsable de la répression catabolique fait encore l'objet de nombreux

débats (Inada *et al.*, 1996; Crasnier-Mednansky, 2008; Gorke & Stulke, 2008; Narang, 2009). Les désaccords sont souvent liés à l'obtention de résultats différents entre les équipes pour une même expérience : par exemple, l'effet de l'ajout d'AMPc exogène sur le regulon Crp en présence de glucose. Tout le monde s'accorde cependant pour dire que les modèles actuels sont incomplets et doivent être révisés. Sans certitude cependant, nous pensons que le mécanisme responsable de la répression catabolique est encore inconnu. Il ne s'agit, pour nous, ni des variations de concentration de l'AMPc intracellulaire ni des variations de l'état de phosphorylation d' $EIIA_{glc}$. Paradoxalement, et c'est ce qui rend les choses si complexes, les rôles du PTS et de l'AMPc sont incontestables.

9 Utiliser la répression catabolique en biologie synthétique

Ainsi nous pensons que le fonctionnement de l'interrupteur, contrôlé par le flux glycolytique et contrôlant l'activité des promoteurs Crp-AMPc dépendants, reste inconnu. Nous avons créé un petit système synthétique qui illustre la fiabilité de cet interrupteur (Figure I.9). Notre système est constitué de deux fusions transcriptionnelles distinctes. La première rapporte l'activité du promoteur *uhpT* via le rapporteur GFP. Ce promoteur est activé par l'inducteur glucose-6-phosphate (G6P). La deuxième fusion rapporte l'activité du promoteur *acs* via le rapporteur luciferase. Le mécanisme d'interrupteur fonctionne de la manière suivante : les bactéries poussent sur un milieu minimum contenant du G6P et de l'acétate. La présence du G6P dans le milieu active le promoteur *uhpT* (ON), ce qui permet l'utilisation de ce sucre comme source de carbone. La mise en place du flux glycolytique empêche l'activité du promoteur *acs* (OFF). Lorsque le G6P est épuisé, le promoteur *uhpT* cesse d'être actif (OFF), le flux glycolytique s'effondre ce qui active le promoteur *acs* (ON). Ce petit système synthétique, bien que basé sur le mécanisme inconnu de la répression catabolique, est robuste, performant et rapide. Nous avons également implémenté une version un peu différente à l'échelle de la cellule isolée. Ce petit détour par la biologie synthétique nous offre une excellente transition pour amorcer la prochaine partie : la description d'un système synthétique de communication intercellulaire par l'AMPc.

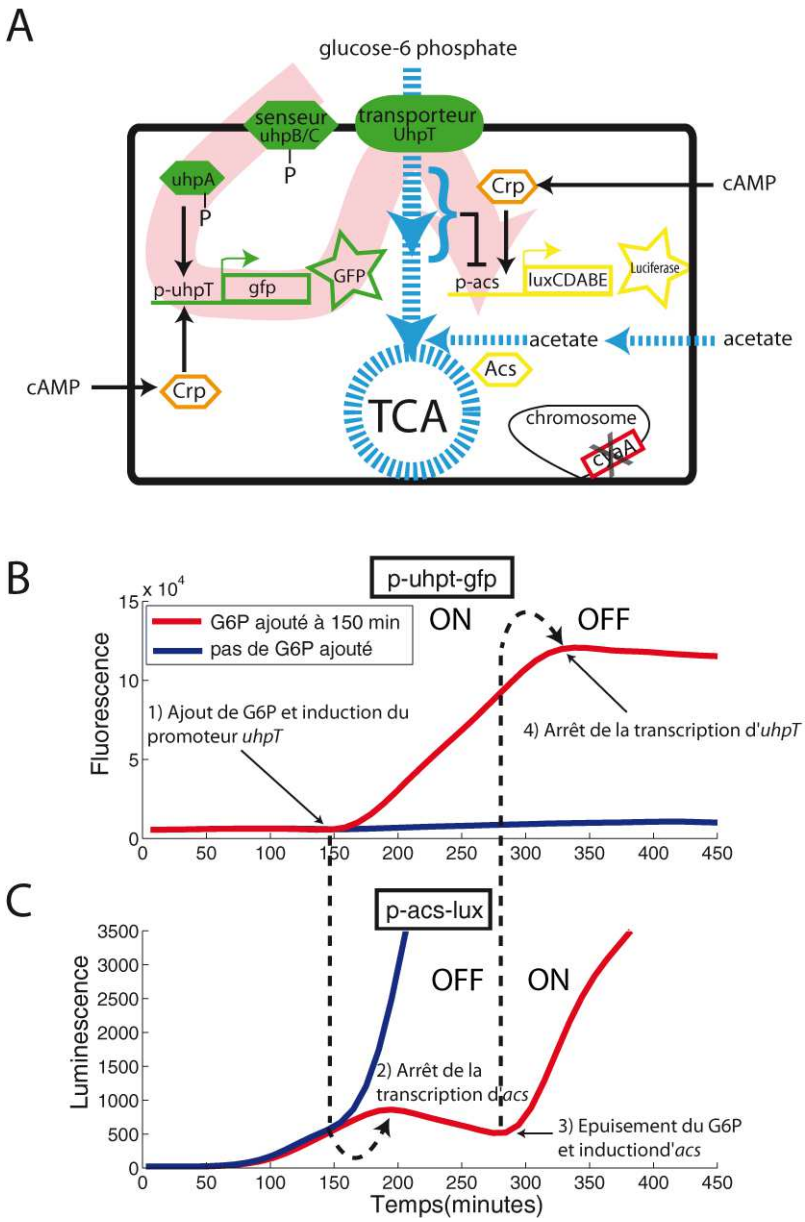

Figure I.9 – Un interrupteur synthétique contrôlé par le flux glycolytique. A. Schéma d'un mutant $\Delta cyaA$ transformé avec deux fusions transcriptionelles (p-acs-lux et p-uhpT-gfp). Le glucose-6-phosphate (G6P) est détecté par un mécanisme à deux composantes (UhpB/UhpA) qui active l'expression du gène *uhpT*. Ce gène code pour le transporteur du G6P. Ce dernier, par le flux glycolytique qu'il génère, stoppe l'expression d'*acs* par le mécanisme inconnu de la répression catabolique. B et C : les bactéries poussent sur un milieu minimum avec du glucose, de l'acétate et de l'AMPc. Au bout de 100 minutes, il y a la première induction du gène *acs* dû à l'épuisement du glucose (C). L'ajout de G6P après 150 minutes déclenche l'expression d'*uhpT* (B). Ceci aboutit à la mise en place d'un fort flux glycolytique qui stoppe l'expression d'*acs* (C). Une fois épuisé, le G6P ne peut plus activer l'expression d'*uhpT* (B). Le flux glycolytique chute et une nouvelle induction d'*acs* se produit (C).

10 Système synthétique de communication intercellulaire par l'AMPc

Pour comprendre le mécanisme responsable de la répression catabolique, nous avons développé de nombreux outils (fusions transcriptionnelles, vecteurs de sur-expression inductibles, construction de souches mutantes). Cependant, ces efforts ne nous ont pas permis de percer à jour le mécanisme responsable de la répression catabolique. En échange, la nature nous a accordé la « serendipity » c'est-à-dire la découverte au hasard qui rend heureux. En plaçant en contact un mutant Δcrp et un mutant $\Delta cyaA$, nous nous sommes rendu compte que ces deux souches réussissaient à communiquer grâce à l'AMPc extracellulaire. L'expérience est relativement simple : nous avons placé une gélose contenant des bactéries Δcrp (dite « sender ») en contact d'une gélose composée des bactéries $\Delta cyaA$ (dite « receiver ») contenant un bio-senseur luminescent très sensible capable de détecter la présence d'AMPc extracellulaire. Ce biosenseur correspond au promoteur *sdh* (Crp-AMPc dépendant) fusionné à l'operon *luxCDABE* (plasmide p-sdh-lux). Après quelques heures, la gélose contenant les bactéries $\Delta cyaA$ s'est mise à émettre de la lumière. L'AMPc produit par la souche « sender » a diffusé dans la seconde gélose puis a pénétré dans les bactéries $\Delta cyaA$, s'est fixé à Crp ce qui a permis l'activation du promoteur *sdh* puis l'émission de lumière (Figure I.10A). Pour parfaire la démonstration, nous avons montré que la gélose « receiver » ne s'allume jamais si l'adenylate cyclase (production de l'AMPc) est absente dans la gélose « sender ». De même, nous avons montré que la présence de Crp dans la gélose « receiver » est indispensable au processus de communication. Ces jeux avec le complexe Crp-AMPc nous ont permis d'illustrer « artistiquement », via la communication, le fonctionnement de cette jolie porte AND que constitue le complexe Crp-AMPc (Figure I.10B).

Pour caractériser plus précisément ce nouveau module de communication extracellulaire, nous avons également fait des expériences en milieu liquide. Ces expériences montrent que l'on peut contrôler très finement la communication en faisant varier la concentration d'adenylate cyclase dans les « senders » ou la concentration de Crp dans les « receivers ». Un des avantages du complexe Crp-AMPc c'est qu'il est déjà connecté à de nombreuses autres fonctions cellulaires. Par exemple, un mutant $\Delta cyaA$ souffre d'un défaut de motilité lié à son incapacité à fabriquer des flagelles. Nous avons pu rétablir, par communication intercellulaire, la fabrication de flagelles et donc la motilité de bactéries $\Delta cyaA$ créant de fait une sorte de système de guidage synthétique. Cela montre qu'il est possible de connecter aisément notre système de communication à d'autres modules (physiologiques ou synthétiques) présents dans la cellule. Ce processus de communication est-il physiologique ? L'AMPc secrété par la bactérie a-t-il

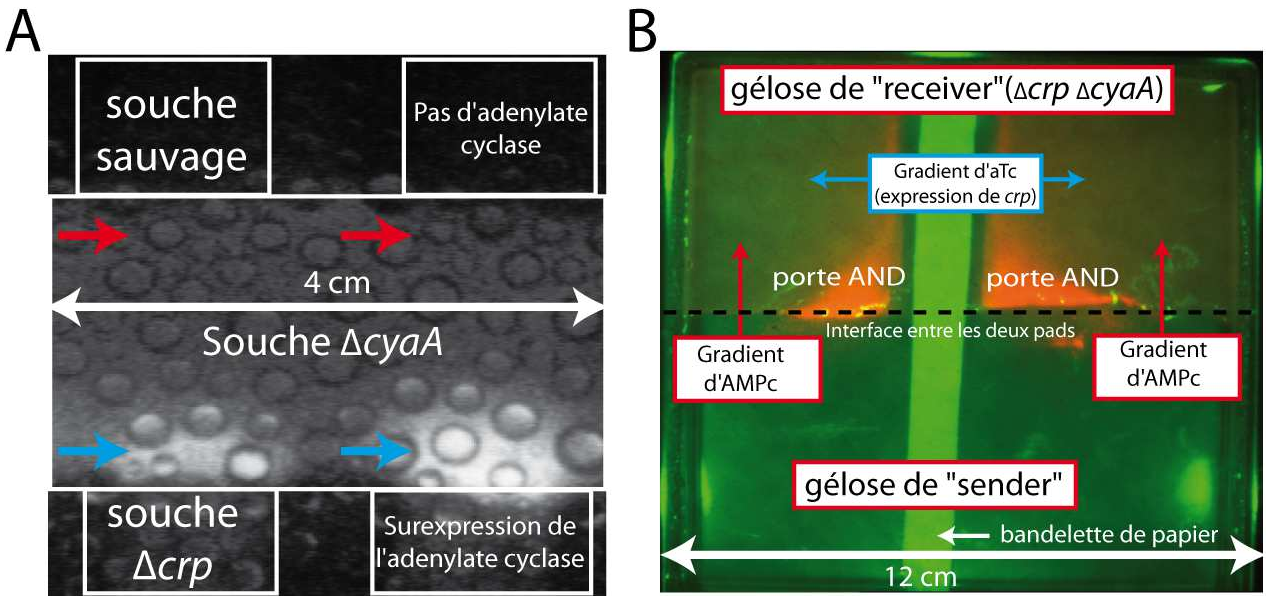

Figure I.10 – Un système de communication intercellulaire via l'AMPc. A. Image de luminescence vue de dessus d'une gélose solide. La photo a été prise dans une chambre noire à l'aide d'une caméra sensible (les ronds sont des gouttes de condensation sur le couvercle). Au milieu de la photo, il y a une longue gélose contenant les cellules « receiver » produisant un niveau basal de lumière. En haut à gauche (flèche rouge), l'interface créée entre la gélose « receiver » et une gélose contenant des bactéries sauvages ne produit pas d'augmentation de lumière. A l'inverse, en bas à droite (flèche bleue), un halo de lumière est observé à l'interface entre la gélose « sender » contenant les bactéries Δcrp et la gélose « receiver ». En bas à droite (flèche bleue), des cellules « sender », sur-exprimant l'adenylate cyclase, déclenchent l'augmentation de lumière dans la gélose « receiver ». A l'inverse, sans expression d'adenylate cyclase, il n'y a pas de halo lumineux visible (en haut à droite, flèche rouge). B. Une photo d'une boite de petri et des données de luminescence (fausse couleur rouge) sont superposées. Une moitié de la boite de petri contient une gélose « sender » produisant de l'AMPc qui diffuse dans la gélose « receiver » (seconde moitié). Au milieu et perpendiculairement à l'interface, une mince bandelette de cellulose saturée avec un inducteur (aTc) permet l'expression de la protéine Crp dans les cellules « receiver ». Deux zones lumineuses (rouges) de part et d'autre de la bandelette apparaissent là où les concentrations d'AMPc et de Crp sont suffisamment élevées pour permettre la formation du complexe Crp-AMPc dans les cellules « receiver » et donc la transcription de la luciferase.

pour fonction la communication ? Nous ne sommes pas encore en mesure de répondre à cette question. Un résultat nous a particulièrement troublé : la limite basse de détection de l'AMPc extracellulaire de notre biosenseur (5-10μM) correspond exactement à la limite haute de production d'AMPc extracellulaire par une souche sauvage (1-10μM). C'est comme si les bactéries sauvages produisaient une concentration d'AMPc extracellulaire juste en dessous du seuil qui commencerait à modifier significativement l'activité du régulon Crp des autres bactéries. Nous n'avons pas d'explications à ce résultat surprenant.

11 Utiliser l'activité luciferase pour inférer l'état métabolique de la cellule

Le rapporteur luciferase a besoin de sources d'énergie cellulaires pour émettre des photons. Cette dépendance est souvent considérée comme un risque d'artefact majeur. Nous allons montrer qu'elle peut aussi être utilisée pour inférer de nombreuses informations sur l'état des cellules. Pour émettre des photons, la luciferase que nous utilisons (*Photorhabdus luminescens*) ne nécessite pas l'ajout de substrat dans le milieu de culture. En effet, ce dernier est produit directement dans la cellule à l'aide de trois enzymes (encodées par *luxC*, *luxD*, *luxE*). La luciferase (encodée par *luxA* et *luxB*) oxydes simultanément ce substrat et le $FMNH_2$ en présence d'oxygène pour émettre un photon à 490 nm. La production de $FMNH_2$ requiert le fonctionnement des réactions centrales du métabolisme du carbone. Ainsi l'émission de lumière n'est pas seulement corrélée à la concentration de la luciferase mais également à l'activité du métabolisme central. Toute perturbation dans cette activité affecte la production de lumière. Cet effet peut être observé lorsque les bactéries épuisent la source de carbone de leur milieu et rentrent en phase stationnaire. A ce moment précis, on observe une chute de l'activité luciferase (que nous appellerons dorénavant ADLA, Abrupt Decline of Luciferase Activity) due à une chute du pouvoir réducteur (Koga *et al.*, 2005). Il est facile de prouver que cette chute est liée à l'épuisement de la source d'énergie car l'ajout dans le milieu, après cet ADLA, de n'importe quelle source de carbone utilisable par les bactéries, rétablit quasi immédiatement l'émission de lumière (Figure I.11).

Pour pouvoir étudier cette dépendance énergétique de la luciferase, nous nous sommes tout d'abord affranchi de toutes régulations transcriptionnelles. Nous avons fusionné un promoteur synthétique indépendant (tet) à l'operon *luxCDABE* (plasmide p-tet-lux). Avec cette construction, l'expression de la luciferase est sous notre contrôle : toutes les variations mesurées dans le signal de luminescence proviennent de changements d'états métaboliques. En faisant pousser des bactéries *E. coli* sauvages transformées avec ce senseur métabolique dans un milieu minimum contenant du glucose et de l'acétate, on observe deux phénomènes intéressants (figure I.12A) :

⇒ il y a une légère diminution transitoire de luminescence lors de la transition glucose-acétate. Cet événement suggère une diminution transitoire du fonctionnement du métabolisme central.

⇒ il y a une chute brutale de l'activité luciferase lorsque l'acétate est épuisé, c'est-à-dire lorsqu'il n'y a plus aucune source de carbone dans le milieu.

Cette méthode de mesure de l'état métabolique des cellules à l'aide d'un « biosenseur » est

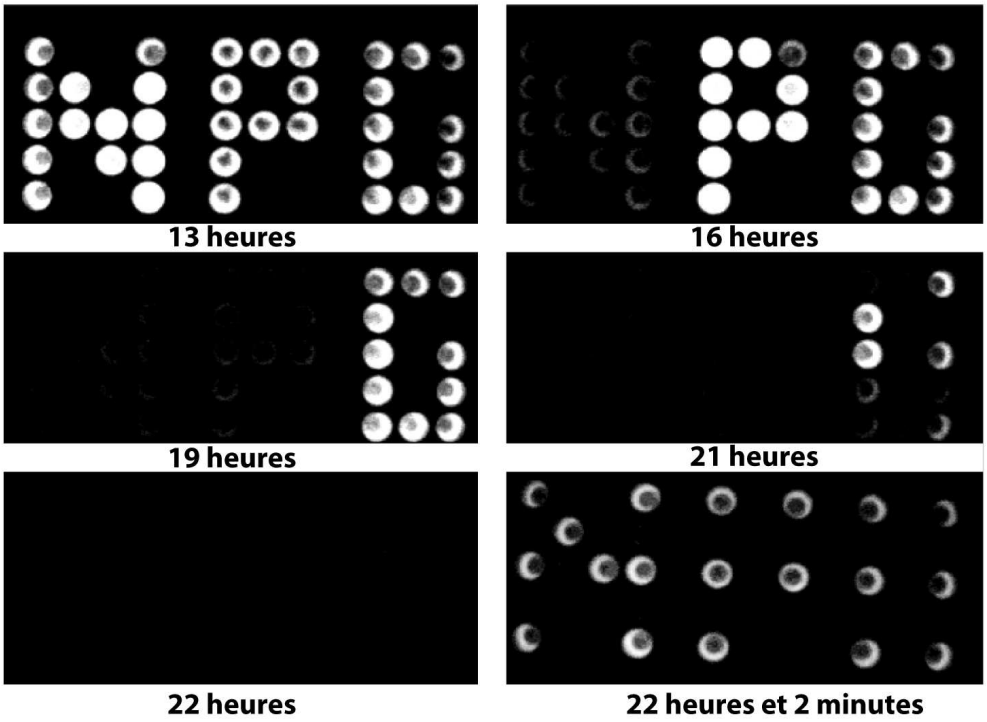

Figure I.11 – La dépendance énergétique de la luciferase. Images de luminescence d'une microplaque 96-puits vue de dessus. Les lettres N, P, G sont formées par le regroupement de plusieurs puits. Chaque puits contient un milieu minimum avec du glucose et des bactéries *E. coli* exprimant l'operon *luxCDABE*. La concentration du glucose augmente de la gauche vers la droite. Par conséquent, sa consommation prend de plus en plus de temps. Quand la source de carbone externe est épuisée, la production d'énergie s'arrête et les lettres s'éteignent les unes après les autres (fonction de la concentration de glucose). L'ajout de glucose dans le milieu (ici dans un puits sur deux) permet le retour immédiat (∼minutes) du signal de luminescence ce qui démontre que les enzymes « luciferases » sont toujours présentes dans la cellule.

intéressante mais elle ne dévoile toute sa puissance que lorsqu'elle est couplée à une banque de simples mutants. Nous avons donc transformé des centaines de mutants avec notre biosenseur à la recherche de signaux qui diffèrent de la souche sauvage.

A titre d'exemple, le profil de luminescence d'un mutant *sucB*, déleté d'un gène codant pour une enzyme du cycle de Krebs, montre une chute brutale de l'activité luciferase non pas lors de l'épuisement de l'acétate (comme c'est le cas pour la souche sauvage) mais lors de l'épuisement du glucose (figure I.12B). En effet, ce mutant ne peut pas utiliser l'acétate comme source de carbone car la néoglucogenèse est coupée. Une souche *sdhB* est aussi déletée d'un gène codant pour une enzyme du cycle de Krebs : on observe aussi une chute de l'activité luciferase lorsque le glucose est épuisé. Cependant l'ampleur de cette chute est bien moins prononcée que celle observée avec le mutant *sucB*. Il est très probable que cette différence de phénotype soit significative et mette en exergue l'existence d'une petite fuite métabolique permettant de

maintenir un fonctionnement minimal du cycle de Krebs et donc une petite activité luciferase dans le mutant *sdhB*. Ainsi nous pensons que la capacité à maintenir l'activité luciferase associée à la forte sensibilité de détection de la lumière permettrait peut-être de détecter et de quantifier des variations fines dans les flux métaboliques. Menée de manière systématique (grand nombre de mutants), cette manière de procéder pourrait peut-être permettre de redessiner les flux métaboliques de manière plus quantitative dans une condition donnée.

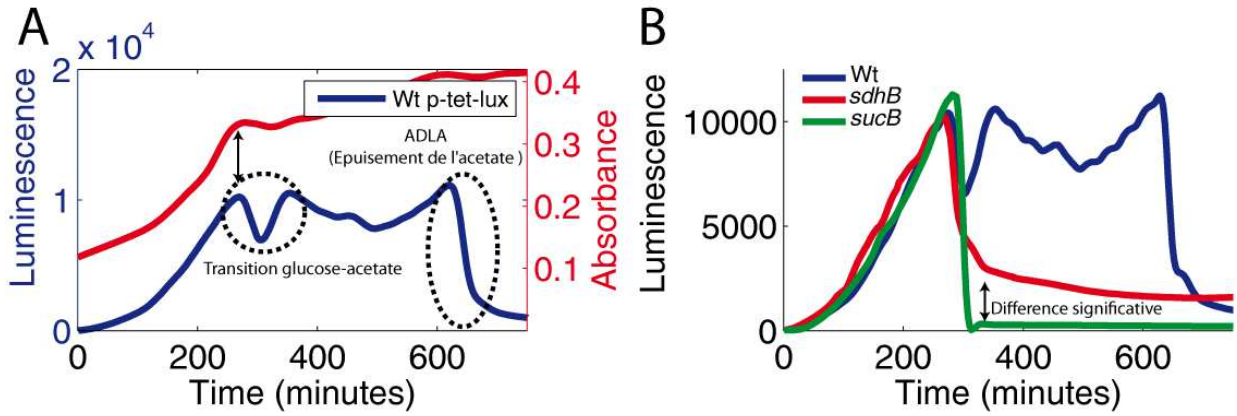

Figure I.12 – Capacité à maintenir l'activité luciferase durant la croissance sur glucose et acétate dans différent mutants. A. Une souche sauvage transformée avec le plasmide p-tet-lux pousse dans un milieu minimum avec du glucose, de l'acétate et l'anhydrotetracycline (l'inducteur du promoteur tet). Quand le glucose est épuisé, il y a une légère diminution suivi du rétablissement de l'activité luciferase (courbe bleue). En effet, cet événement survient au même moment que la cassure visible sur la courbe de croissance (courbe rouge). Plus tard, lorsque l'acétate est épuisé, il y a une chute brutale de l'activité luciferase. B. Dans un mutant *sucB*, cette chute brutale de lumière n'a pas lieu lors de l'épuisement de l'acétate mais lors de l'épuisement du glucose. En effet, la délétion du gène *sucB* empêche la néoglucogenèse et donc la possibilité d'utiliser l'acétate comme source d'énergie. Dans un mutant *sdhB*, la capacité à maintenir l'activité luciferase est sévèrement diminuée par rapport à la souche sauvage mais significativement supérieure au mutant *sucB* : il subsiste sans doute une petite fuite métabolique permettant quand même l'utilisation de l'acétate comme source d'énergie.

La capacité à maintenir l'activité luciferase permet de se focaliser sur une enzyme, un flux spécifique. Ainsi nous avons pu mesurer, en temps réel, l'activité relative d'une enzyme : l'acétyl-coenzyme A synthétase encodée par le gène *acs*. L'idée consiste à créer et contrôler un goulot d'étranglement métabolique dans des bactéries saturées en luciferase. Nous avons donc supprimé toute trace d'acétyl-coenzyme A synthétase dans des bactéries placées dans un milieu minimum avec de l'acétate. Dans ces conditions, ces bactéries ne peuvent pas utiliser l'acétate : le métabolisme central est inactif. La luciferase, pourtant présente en forte concentration, ne peut pas émettre de photons. Puis nous rouvrons « la vanne » lentement : l'arrivée des premières enzymes d'acetyl-coenzyme A synthétase réamorce le métabolisme central qui peut alors utiliser l'acétate comme source d'énergie : la brusque remontée du pouvoir réducteur entraine

le rétablissement de l'activité luciferase et le niveau de ce rétablissement est directement corrélé à la concentration de l'acetyl-coenzyme A synthétase. Avec cette méthode, il est donc possible de connaître son activité relative, directement *in vivo* et en temps réel.

Enfin, l'utilisation du promoteur *acs* fusionné à l'operon *luxCDABE* (plasmide p-acs-lux) nous a permis d'inférer certains paramètres extérieurs, encore une fois directement et en temps réel. Nous avons vu que l'épuisement du glucose lève la répression exercée sur ce promoteur. Dans un milieu contenant du glucose et de l'acétate, l'induction de luminescence indique donc l'épuisement du glucose (Figure I.13A). A l'inverse, la chute brutale de l'activité luciferase signale l'épuisement de l'acétate (Figure I.13B). En associant ces deux événements, nous pensons qu'il est possible de mesurer la quantité relative d'acétate secrété par les bactéries dans le milieu c'est-à-dire le débordement du métabolisme (overflow metabolism). Lorsque les bactéries poussent sur glucose, le métabolisme central est saturé et une partie de l'énergie est évacuée vers l'extérieur sous forme d'acétate en vu d'une utilisation future. Pour mesurer ce phénomène, nous avons fait pousser des bactéries dans un milieu contenant uniquement du glucose. Nous avons remarqué que le temps entre l'induction de la luciferase (épuisement du glucose) et la chute de l'activité luciferase (épuisement de l'acétate) augmente proportionnellement avec la concentration de glucose utilisée (Figure I.13C). L'explication la plus raisonnable pour expliquer l'accroissement de ce délai est l'augmentation de la concentration extérieure d'acétate. Pour étayer cette hypothèse, nous avons testé des mutants (*ptsG*, *pgi*) qui ralentissent la consommation du glucose (et donc la glycolyse) ce qui limite le débordement du métabolisme. Dans ces mutants et pour une même concentration de glucose, le délai entre l'induction de la luciferase et la chute de l'activité luciferase est beaucoup plus court comparé à une souche sauvage (Figure I.13D). Ce résultat suggère, comme escompté, que la sécrétion d'acétate dans ces mutants est d'une ampleur beaucoup plus limitée.

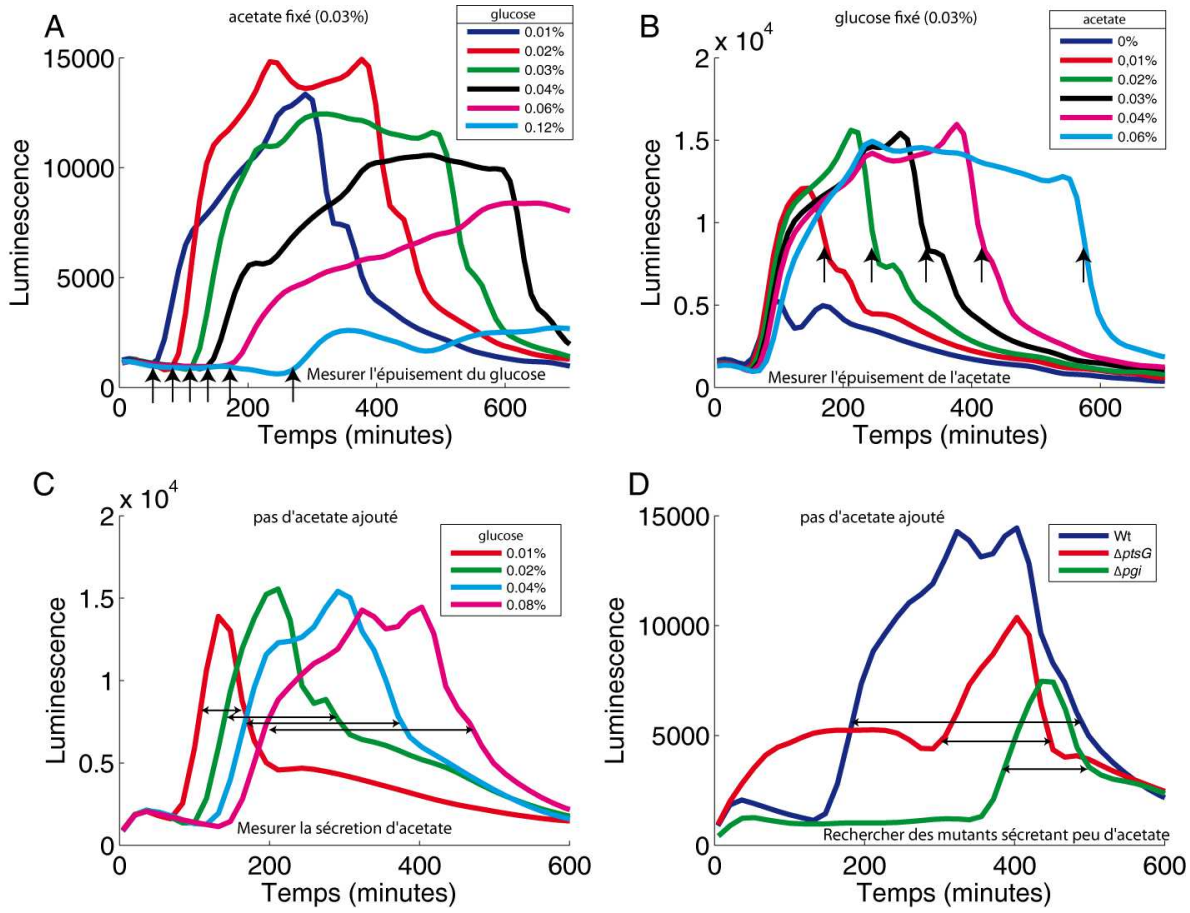

Figure I.13 – Un outil pour mesurer la concentration extérieure de glucose, d'acétate et le débordement du métabolisme. A. Le plasmide p-acs-lux peut être utilisé pour mesurer l'épuisement du glucose (la concentration d'acétate est fixée). En effet, une forte augmentation de luminescence se produit lorsque le glucose est épuisé (de manière dose-dépendante). B. Le plasmide p-acs-lux peut être utilisé pour mesurer l'épuisement de l'acétate (la concentration du glucose est fixée). En effet, le temps auquel survient la chute de lumière est proportionnel à la concentration d'acétate. C. Des bactéries poussent dans un milieu avec du glucose mais sans acétate. Quand la concentration de glucose augmente, le délai entre l'induction de la luminescence (épuisement du glucose) et la chute brutale de la luminescence (épuisement de l'acétate) augmente proportionnellement. Ce délai pourrait mettre en évidence l'acétate sécrété dans le milieu dû au débordement du métabolisme durant la croissance sur glucose. D. Pour une même concentration de glucose, des mutants avec un flux glycolytique réduit (*ptsG*, *pgi*) ont un délai entre l'induction de la luminescence et sa chute brutale qui est réduit par rapport à celui observé avec la souche sauvage. La réduction de ce délai pourrait mettre en exergue une diminution de la sécrétion d'acétate et donc un débordement du métabolisme plus faible.

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Chapitre II

A genome-wide screen for identifying all regulators of a target gene

A genome-wide screen for identifying all regulators of a target gene

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Abstract

We have developed a new screening methodology for identifying all genes that control the expression of a target gene through genetic or metabolic interactions. The screen combines mutant libraries with luciferase reporter constructs. Instead of a static picture of gene expression, this method allows dynamical monitoring in different environmental conditions. Mutants with interesting phenotypes can thus be selected based on multiple criteria, and the expression dynamics of the target gene can be extensively characterized. We apply the method to identify the genes that control the expression of the *acs* gene of *Escherichia coli*. We confirm most of the known genetic regulators and identify new regulatory influences, many of which involve metabolic intermediates or metabolic sensing. An analysis of mutants involved in glycolysis and glucose transport demonstrates that the classical model of catabolite repression in *E. coli* needs to be amended.

Introduction

The adaptation of bacteria to changes in their environment is controlled on the molecular level by a large and complex regulatory network involving genes, mRNAs, proteins and metabolites (Jacob & Monod, 1961). Understanding, and therefore eventually predicting, the dynamics of this regulatory network is a general goal of biology, and in particular of the field of systems biology. The basic ingredient of this type of research is knowing the topology of the underlying regulatory network. An increasing number of such regulatory connections are documented in bioinformatics databases such as EcoCyc (`http://EcoCyc.org`), which lists more than 5345 regulatory interactions for *E. coli* (Keseler *et al.*,

2010).

Different methods are used to identify and characterize these interactions. Evidence for specific interactions can come from in vitro measurement of physical DNA-protein interactions (footprinting, gel retardation assay). Computational tools can predict interactions based on consensus sequence. Transcriptomics (DNA microarrays) is a popular approach to study transcription on a global scale (Brown & Botstein, 1999). Typically, this technique allows detecting the targets of a particular regulator by studying the effect of the deletion of this regulator on the expression of all genes of a genome. ChIP-Chip is another global approach, identifying direct interactions of a specific transcription factor through the detection of chromosome-wide DNA binding (Grainger & Busby, 2008). All these tools aim at identifying regulons, i.e., all the targets of a given regulator (Neidhardt & Savageau, 1996). However, to our knowledge, no global method exists to determine the regulators that control, directly or indirectly, the expression of a particular target gene. We present such a method in this manuscript.

We use the GFP and luciferase reporters to measure promoter activities in living cells (Van Dyk *et al.*, 2001; Hakkila *et al.*, 2002). These constructs provide not only a static picture of expression levels, but allow measuring the dynamics of gene expression with a high temporal resolution (Zaslaver *et al.*, 2006). The luciferase system is particularly useful since it allows measuring gene expression in a colony growing on solid medium. We have developed a technique for efficiently transforming 3571 *E. coli* single-gene knockout mutants (Baba *et al.*, 2006) with the appropriate reporter plasmid. The screening strategy consists in comparing the luminescence of mutant colonies with the luminescence of a wild-type colony: a significant difference suggests that the deleted gene controls, directly or indirectly, the expression of the target gene.

We have applied this method to identify regulators that control the expression of the *acs* gene, coding for the acetyl coenzyme A synthetase (Kumari *et al.*, 1995). This enzyme converts acetate to acetyl coenzyme A. We have chosen this gene for three reasons. First, this enzyme plays a key role in the "acetate switch": when bacteria grow rapidly on glucose, they excrete acetate which is subsequently used as a carbon source when glucose is exhausted (Wolfe, 2005). Acetyl coenzyme A synthetase is thus at the center of an inversion of metabolic flow from glycolysis to neoglucogenesis. Second, the accumulation of acetate in the culture medium is an important problem in industrial fermentations since this organic acid inhibits cell growth and recombinant protein production. Different approaches are currently developed to reduce acetate accumulation by modifying central metabolic pathways (Gosset, 2005). Lastly, the *acs* gene

was also selected because its complex regulation has been extensively described and thus allows a thorough validation of the method (Kumari *et al.*, 2000; Sclavi *et al.*, 2007; Shin *et al.*, 2009). Briefly, transcription of this gene is repressed by the nucleoproteins Fis and IHF and positively regulated by the CRP-cAMP complex. The latter activation implies the well-studied carbon catabolite repression mechanism where the glucose concentration controls the formation of the CRP-cAMP complex by adjusting the cAMP production via the phosphotransferase system (PTS) (Gorke & Stulke, 2008a). The *acs* gene is thus at the end of a well-known, complex regulatory cascade, an ideal property for our purpose. Our screen confirms known regulatory mechanisms of *acs* expression. We also find new candidates for regulatory input that highlight the tight links between metabolism and gene expression.

Results

A screening strategy for identifying the regulators of *acs*

Our screening strategy is outlined in Fig. 1. To measure *acs* promoter activity we have constructed a reporter plasmid containing a transcriptional fusion of the *acs* promoter region (394 bp) to the *luxCDABE* operon. This operon codes for the heterodimeric luciferase protein (*luxAB*) and the enzymes (*luxCDE*) necessary for producing the luciferase substrate, a long-chain aldehyde (Meighen, 1991). We have chosen this reporter system because of the highly sensitive luminescence signal, the absence of background, the continuous production of bioluminescence without added substrate, and the possibility to measure luminescence at the colony level. The reporter plasmid was transformed into the 3911 single-gene knockouts of *Escherichia coli* (Keio collection) using our high-throughput transformation procedure (see Methods). Transformation efficiency was 91%: we have thus obtained 3571 transformants. We spotted the transformed bacteria on solid media and acquired the luminescence images of the colonies that appeared on the plates.

The luminescence of the colonies changes as a function of culture time and growth condition. The powerful features of our screen are that we can monitor the dynamics of gene expression at the colony level (see an example in Supplementary Information 2: Movie) and adapt growth conditions to be most relevant to the gene under investigation. The *acs* promoter is activated after exhaustion of the preferred carbon source. We therefore measured the luminosity of colonies after 24h and 48h of growth on two different media: rich LB medium and minimal glucose medium. We thus obtained four complete sets

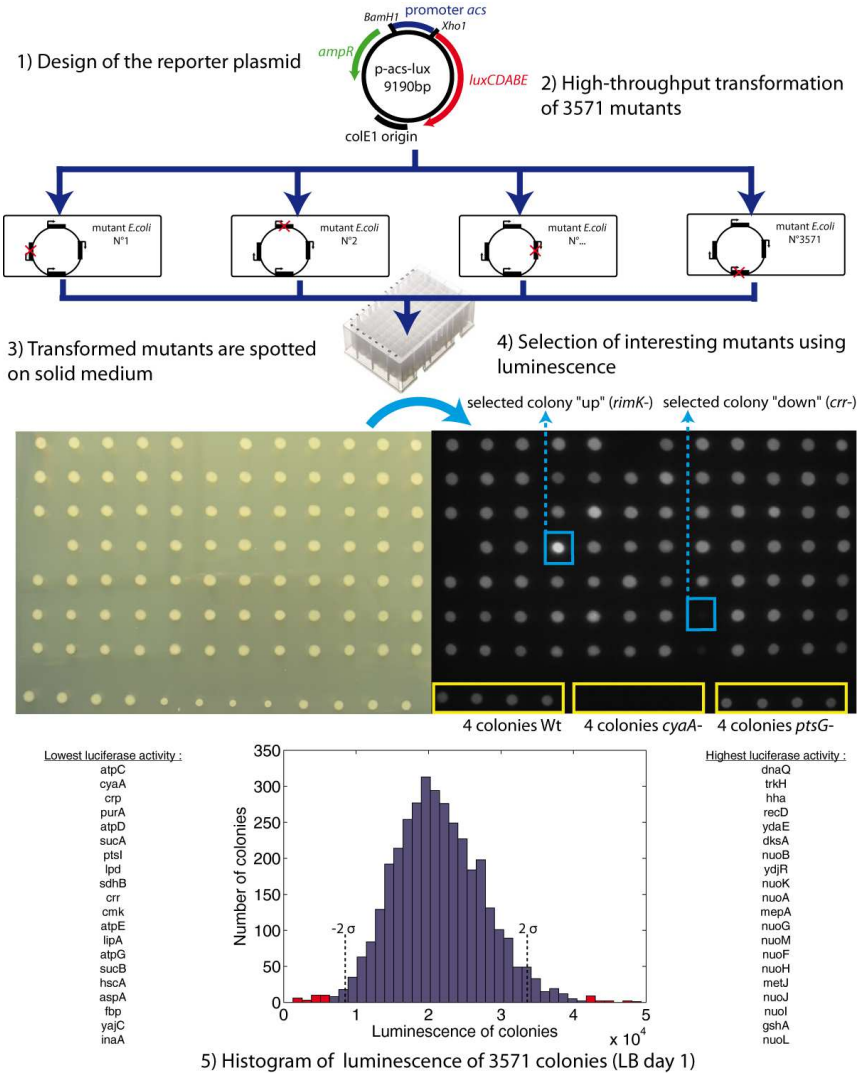

Figure 1. A screening strategy for identifying new regulators. The *acs* promoter region was cloned upstream of the *luxCDABE* operon on a low-copy number plasmid. This plasmid is transformed into 3571 single knock-out mutants of *E. coli* (top). The transformed bacteria were spotted onto solid medium and the luminescence of the colonies was quantified (LB-agar day 1) (middle). The histogram (bottom) shows the distribution of luminescence emitted by all colonies. The names of the 20 mutants with the highest/lowest luciferase activity (in red on the histogram) are listed on the sides. In these mutants, light emission is significantly different from the wild-type (0.95 confidence), as determined by fitting a normal distribution to the histogram and noting that the mean of the distribution corresponds to wild-type levels. The broken vertical lines indicate two standard deviations above and below the mean. Among the mutants with the lowest luciferase activity, we find 7 genes (*atpC, atpD, atpE, atpG, sucA, sucB*, *sdhB, lpd*A) involved in energy supply and four genes involved in catabolite repression (*cyaA*, *crp*, *crr*, *ptsI*). Among the mutants with the highest luciferase activity, our screen detects a transcriptional regulator (*hha*) and 10 genes of the *nuo* operon.

of luminosity images of the plates. We quantified the luminosities of the 3571 colonies after 24h of growth on LB plates (Supplementary Data). Fig. 1 shows the corresponding distribution of luminosities. Automatic quantification was not possible for the other growth conditions, but the complete dataset is available in Supplementary Data. From these four batches of luminescence pictures, we have selected 517 (15%) colonies for a detailed kinetic analysis. We also include data about the growth phenotype: among the 3571 transformants, eighty auxotrophic mutants did not grow on minimal medium-agar-glucose (Supplementary Information 4).

The dynamics of *acs* expression

For the 517 pre-selected genes, we measured the dynamics of *acs* expression in liquid cultures grown in a minimal medium containing glucose and acetate in order to focus on the physiologically important transition between those two carbons sources. The expression patterns were measured in an automated microplate reader and analyzed as previously described (de Jong *et al.*, 2010). Fig. 2A shows the expression profile of *acs* in the wild-type strain. We observe a sharp increase of *acs* transcription when glucose is exhausted. This expression pattern is very robust and independent of the reporter system since we observe an identical pattern with the *gfp* reporter carried on a plasmid with a completely different replication mechanism (Fig. 2B). Note that luminescence is largely preferable for screening because bacteria have a significant auto-fluorescence background, probably due to flavin mononucleotide or riboflavin (Billinton & Knight, 2001).

Known regulators

An important criterion for the validity of a screen is that known interactions should be confirmed. The *acs* gene is positively regulated by the CRP-cAMP complex (*crp* gene) and thus by the well-studied carbon catabolite repression mechanism. The influx of glucose through the phosphotransferase system (PTS) controls adenylate cyclase (*cyaA* gene) activity and thus cAMP production. The PTS is composed of enzymes EI (*ptsI* gene), HPR (*ptsH* gene), $EIIA_{glc}$ (*crr* gene) and $EIIB/C_{glc}$ (the glucose transporter encoded by the *ptsG* gene). When glucose is exhausted, the four PTS enzymes are phosphorylated and $EIIA_{glc}$ binds to and activates adenylate cyclase. The cAMP concentration quickly rises and the Crp-cAMP complex activates target promoters such as the *acs* promoter (Gorke & Stulke, 2008a) (Fig. 5).

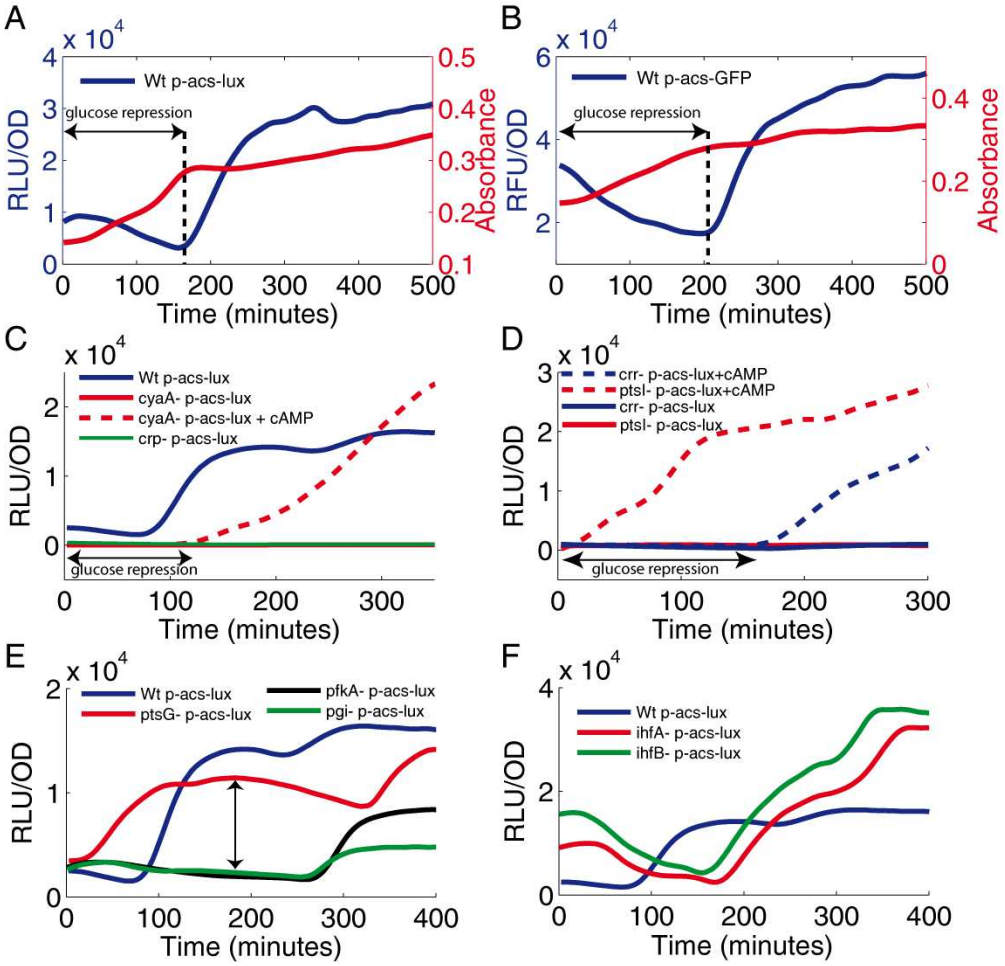

Figure 2. Dynamics of gene expression of known mutants in a liquid minimal medium containing glucose and acetate. A. The expression profile of the *acs* luciferase reporter plasmid shows that *acs* expression is induced when glucose is exhausted. B. The *acs* expression profile using GFP as a reporter is identical to the profile observed with luciferase (A). C. *acs* expression is completely absent in *crp* and *cyaA* strains. cAMP added to the medium (5mM) at the beginning of the experiment complements the *cyaA* strain but glucose repression is maintained. D. Very low expression of *acs* in the *crr* and *ptsI* strains. cAMP added to the medium at the beginning of the experiment directly complements the *ptsI* strain during growth on glucose, whereas the *crr* strain only responds once glucose is exhausted. E. In the *ptsG* mutant, *acs* induction is delayed because glucose is consumed more slowly. Moreover, *acs* expression during glucose consumption is much stronger than in the wild-type strain. The glycolysis mutants *pgi* and *pfkA* also grow more slowly, but *acs* expression remains very low in exponential phase. F. The expression profiles of the *ihfA* and *ihfB* strains show stronger *acs* expression than the wild-type during growth on acetate. The highly similar expression kinetics of the two mutants (with genes located at different sites on the chromosome) shows the high internal consistency of the method.

The analysis of the genes involved in glucose repression confirms that our screen reliably identifies dramatic, but also subtle changes in the regulation of the target gene. In *cyaA* and *crp* strains, there is no transcription of *acs* at all, demonstrating the strict dependence of *acs* transcription on the CRP-cAMP complex. The activity of the *acs* promoter can be restored in the *cyaA* strain by adding cAMP to the medium but, interestingly, only after glucose exhaustion (Fig. 2C). The *crr* and *ptsI* strains show the expected phenotype: a very low *acs* expression, although significantly higher than in the *cyaA* and *crp* strains. cAMP added to the medium clearly restores *acs* expression in these two strains confirming that these mutants cannot activate adenylate cyclase. As in the *cyaA* strain, cAMP fails to complement the *crr* strain while glucose is still present in the growth medium. In contrast, the *ptsI* strain is directly complemented by cAMP even in presence of glucose (Fig. 2D). This difference of reactivity is not explained by the current model of catabolite repression: adding cAMP to the medium should bypass the glucose repression. Consequently, the *cyaA* and *crr* mutants should behave as the *ptsI* strain, with a direct activation of *acs* even in presence of glucose. The screen has thus revealed new subtleties of a well characterized control system.

Expression of *acs* is reported to be negatively regulated by two histone-like proteins Fis and IHF (Browning *et al.*, 2004). The Fis protein is abundant in exponential phase (Ali Azam *et al.*, 1999). Since *acs* expression is already very low in this growth phase, the observed effect of the *fis* deletion is rather subtle: the *acs* expression profile is rather similar to that of the wild-type strain (data not shown). IHF acts mainly in stationary phase (Wolfe, 2005) and we accordingly observe a higher *acs* expression in late stationary phase (Fig. 2F). The perfect superposition of *acs* expression profiles observed in the *ihfA* and *ihfB* strains (each coding for a subunit of IHF and transcribed from different chromosomal locations) confirms the high accuracy and internal consistency of the method.

Metabolic influences on the reporter system

For the purpose of our screen, we want luciferase to report only the changes in gene expression of the target gene. However, the emission of light produced by the colonies does not only depend on *luxCDABE* transcription, but is also influenced by the metabolic state of the cell, such as concentrations of oxygen, $FMNH_2$, ATP, and NADPH. These metabolites are involved in the enzymatic reactions that lead to light emission (Koga *et al.*, 2005). The *nuo* operon (13 genes) is a striking example of these metabolic effects on luciferase activity: all corresponding colonies show a higher luminescence on LB plates (Fig. 3A).

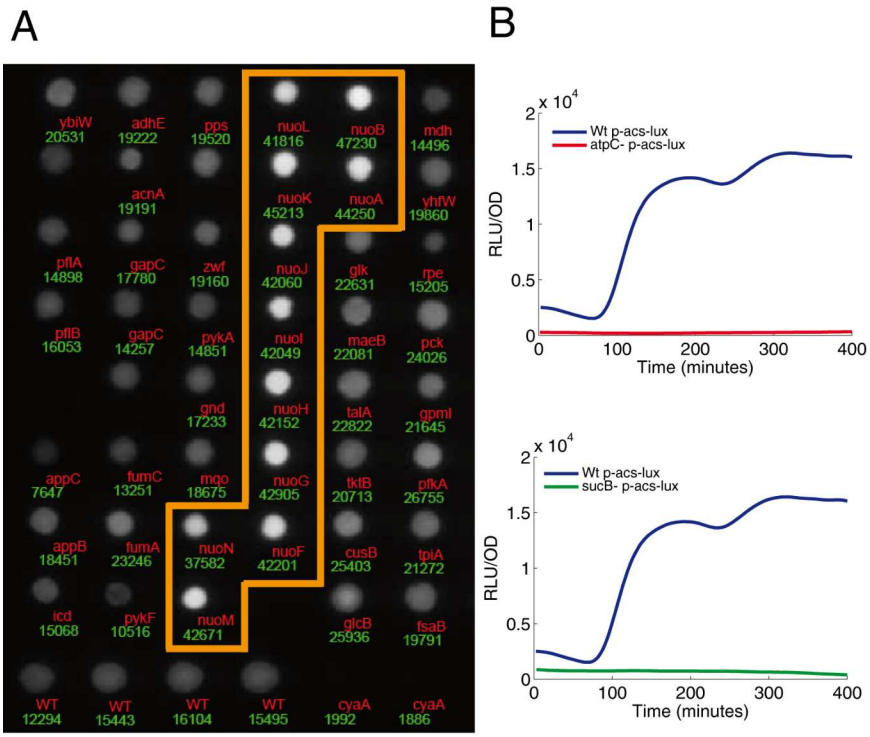

Figure 3. Luciferase and the energy supply. A. Higher level of luminescence of different mutants of the *nuo* operon. This effect is probably due to the dependence of the luciferase reaction on reducing power. The *nuo* operon codes for the NADH dehydrogenase involved in the respiratory chain. In the absence of a functional enzyme, reducing power accumulates and thus stimulates the luciferase reaction. The uniform increase in luminescence of all genes in the operon illustrates the high reproducibility of the screen. B. The *atpC* and *sucB* mutants do not induce *acs* (producing light) when glucose is exhausted.

The *nuo* operon codes for the NADH dehydrogenase involved in the respiratory chain. In the absence of a functional enzyme, reducing power accumulates (Pruss *et al.*, 1994), thus stimulating the luciferase reaction. This phenotype of the *nuo* mutants was not observed during the kinetics in liquid medium. In fact, it is the combination of a specific deletion with a specific medium that leads to this metabolic artifact. Consequently, it is not possible to generate a universal list of false positive mutants.

However, a systematic way to detect and eliminate false positives due to the reporter system is to validate the results with a different reporter system that is independent of the metabolic state of the cell. We therefore transformed the 517 selected mutants with the *gfp* reporter plasmid, also carrying a different origin of replication (thus controlling for mutants that would affect the plasmid copy number). We acquired the expression profile of *acs* for all selected mutants using the two reporter systems. Fig. 4A shows, for each reporter, the strength of *acs* induction, computed from the area under the induction curve. Mutants for which *acs* activation is very different in the two reporter systems (points far off the diagonal) likely represent artifacts of at least one of the reporter systems. We report, for example, 14 false positive mutants (at the left border of the scatterplot), probably affecting light production rather than diminished *acs* expression.

As a further control for the validity of the reporter assay, we measured transcription for several mutants using quantitative RT-PCR. Mutants in genes coding for the PTS (*ptsG*, *crr*, *ptsI*), cAMP production (*cyaA*), regulatory proteins (*ihfB*) and metabolic enzymes (*lpdA*, *sucB*) precisely reproduce the effects observed with the reporter genes (Fig. 4B).

Metabolic regulation of *acs* expression

Even though part of the PTS, the *ptsG* mutant (which lacks the glucose transporter) behaves very differently from the *ptsI* and *crr* mutants: in the *ptsG* strain, colonies on M9 glucose are much more luminescent than those of the wild-type strain (Supplementary data) and the dynamical analysis shows a higher *acs* expression level in exponential phase as compared to the wild-type strain (Fig. 2E). According to the classical model of carbon catabolite repression, this phenotype reflects an increased intracellular concentration of cAMP in exponential phase as a consequence of the lower rate of glucose influx (Hogema *et al.*, 1998). The deletion of metabolic enzymes (*pgi*, *pfkA*) interrupts glycolysis and forces the metabolic flux to pass though the pentose phosphate pathway. Like the *ptsG* strain, these mutants grow more slowly on glucose (delayed induction of *acs* expression), but in this case, *acs* expression remains completely shut-

off in exponential phase (Fig. 2E).

Several of the mutants with the lowest colony luminescence carry deletions of genes involved in the energy supply of the cell (Fig. 1), such as the genes coding for ATP synthetase (*atpC, atpD, atpE, atpF*) and the genes coding for enzymes of the TCA cycle: the oxoglutarate dehydrogenase complex (*sucA*, *sucB* and *lpdA*) and, to a lesser extent, succinyl-CoA synthetase (*sucCD*) and succinate dehydrogenase (*sdhCDAB*). We found that theses mutants fail to grow on acetate (Supplementary Information 4). In these mutants, the luminescence profile shows an almost total absence of *acs* expression (Fig. 3B). This result may either represent (i) an artifact of the luminescence reporter system or (ii) a genuine regulation of the *acs* promoter. Our current data do not allow us to definitively decide between the two possibilities: the nature of the deleted genes (enzymes involved in the energy supply) strongly suggest a mebabolic artifact but the following observations argue for a genuine regulation: the effect is reproduced with the fluorescence reporter and qRT-PCR (Fig. 4) and expression is absent even in rich medium where energy supply should not play a role (Supplementary Data).

Discussion

A new method for identifying the regulators of *acs*

The classical way to dissect a regulatory network, closely linked to classical approaches in genetics, consists in disrupting a gene of interest and observing the effects on the organism. This approach identifies the downstream targets of the gene of interest. Here, we have developed a method to do the opposite: given a gene of interest, we can identify all factors, genetic and metabolic, that affect the expression of this gene. Our method relies on a high-throughput transformation of a luciferase reporter plasmid into an extensive mutant collection. Even though we have focused on transcriptional regulation, the method can certainly be extended to include translational fusions. We use the Keio-mutant collection, but the scope of the screen could be extended to include, e.g., mutants of non-coding RNAs and intergenic regions that may contain unknown ORFs. We have chosen luciferase as a reporter because it allows easy screening at the colony level without any significant background signal. Furthermore, the luciferase signal of a colony is highly reproducible, with a standard deviation of about 5% for independent colonies of the same mutant. The enzymatic reaction of luciferase depends on cellular factors such as ATP concentration or redox potential. For a given medium, deletions that seriously affect the concentration of these factors may

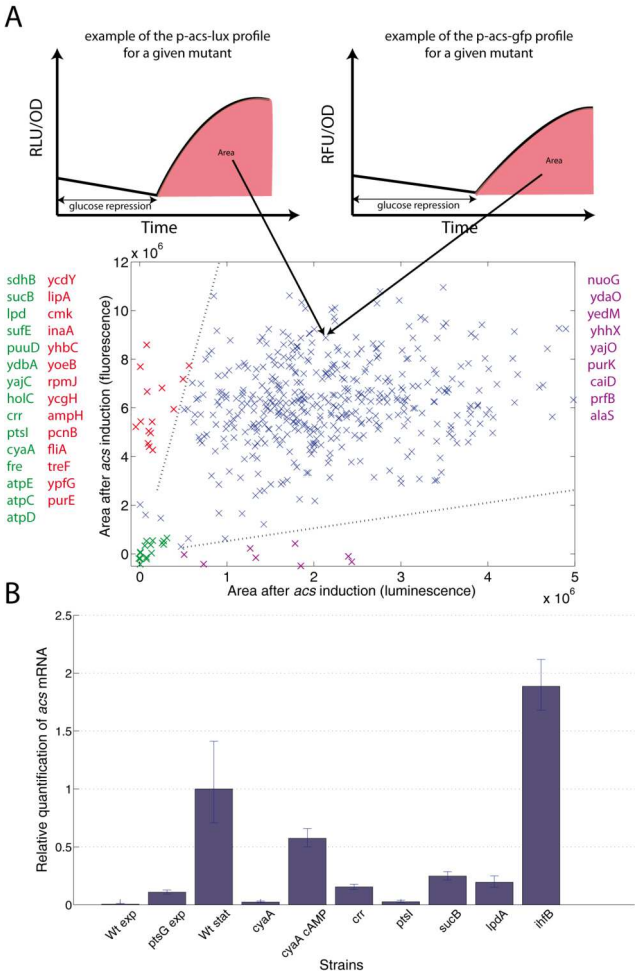

Figure 4. Comparing luminescence, fluorescence and qRT-PCR. A. The area under the expression curve after glucose exhaustion is a good measure of the induction of *acs* expression. The areas were computed for all measured luminescence and fluorescence profiles. The scatterplot shows the correspondence between induction measured by the luminescence and fluorescence reporters. Mutants beyond the dotted lines (four fold deviation from the correlation line) show a significant discrepancy between the two reporter systems. Mutants for which the fluorescence induction is normal, but luminescence low (red), probably affect the luminescence reporter system. Mutants that affect catabolite repression, the TCA cycle, or ATP synthetase show very low fluorescence and luminescence (green). B. Direct measurement of relative mRNA levels of selected mutants using qRT-PCR. All mRNA levels were measured 30 min after glucose exhaustion except for the first two samples, marked Wt exp and *ptsG* exp, that were measured during growth on glucose. All concentrations were normalized to the expression level of the induced wild-type strain. The *acs* mRNA is almost undetectable in the wild-type strain in exponential phase, whereas the *ptsG* mutant shows a clear signal. No *acs* expression is observed in the *cyaA* mutant (*cyaA*), but expression is restored when the medium is complemented with cAMP (*cyaA* cAMP). As expected, the *crr*, *ptsI*, *sucB* and *lpdA* strains show a reduced *acs* expression in comparison with the wild-type strain, and the *ihfB* mutant has a stronger *acs* expression.

generate false positives. However, these can be easily excluded by using an alternative reporter system or verification by qRT-PCR.

An important novel feature of our screen is the possibility to directly measure the dynamics of gene expression during the screen or by high-throughput measurements of selected mutants in an automated plate reader. Furthermore, the original screen, as well as these additional analyses, can be carried out in many different environmental and growth conditions.

The regulation of *acs* transcription

The effects of some interesting mutants identified by the screen are summarized in Fig. 5. We have confirmed known interactions that regulate the transcription of *acs* and we have discovered interesting new directions to explore. The *acs* promoter activity is mainly controlled by the CRP-cAMP complex: transcription is completely abolished without CRP or cAMP. We also confirm the involvement of the phospho-transferase system which sets the intracellular concentration of cAMP: the *crr* and *ptsI* mutants have a very limited *acs* expression except when cAMP is added to the medium. Even though part of the same glucose uptake machinery, the *ptsG* mutant behaves differently from the other PTS mutants: it does not require the addition of exogenous cAMP to activate the *acs* expression.

We failed to induce *acs* expression in *cyaA* or wild-type strains by adding cAMP while glucose was still present in the growth medium. Those results could possibly be explained by another well-known mechanism of glucose repression: inducer exclusion. In this control system, the dephosphorylated form of $EIIA_{glc}$, the dominant form in the presence of glucose, binds and inhibits different sugar transport systems, thus preventing the entry of the inducing sugar (Roseman & Meadow, 1990) into the cell. However we do not believe that inducer exclusion can account for the glucose repression of the *acs* transcription in the presence of exogenous cAMP. To date, no inducer of *acs* expression has been described and acetate can enter the cell by passive diffusion (Gimenez *et al.*, 2003). Furthermore, the central component of inducer exclusion is $EIIA_{glc}$, encoded by the *crr* gene. Yet, we still observe glucose repression in a *crr* mutant in the presence of exogenous cAMP (Fig. 2D). This experiment demonstrates that neither variations of cAMP concentration nor variations of the phosphorylated state of $EIIA_{glc}$ are sufficient to explain the glucose repression that we observe. Consequently, and paradoxically, our results contradict the current two main models of carbon catabolite repression while confirming the involvement of the CRP-cAMP complex and the PTS in the transcription of the *acs* gene. Despite more than four decades of research,

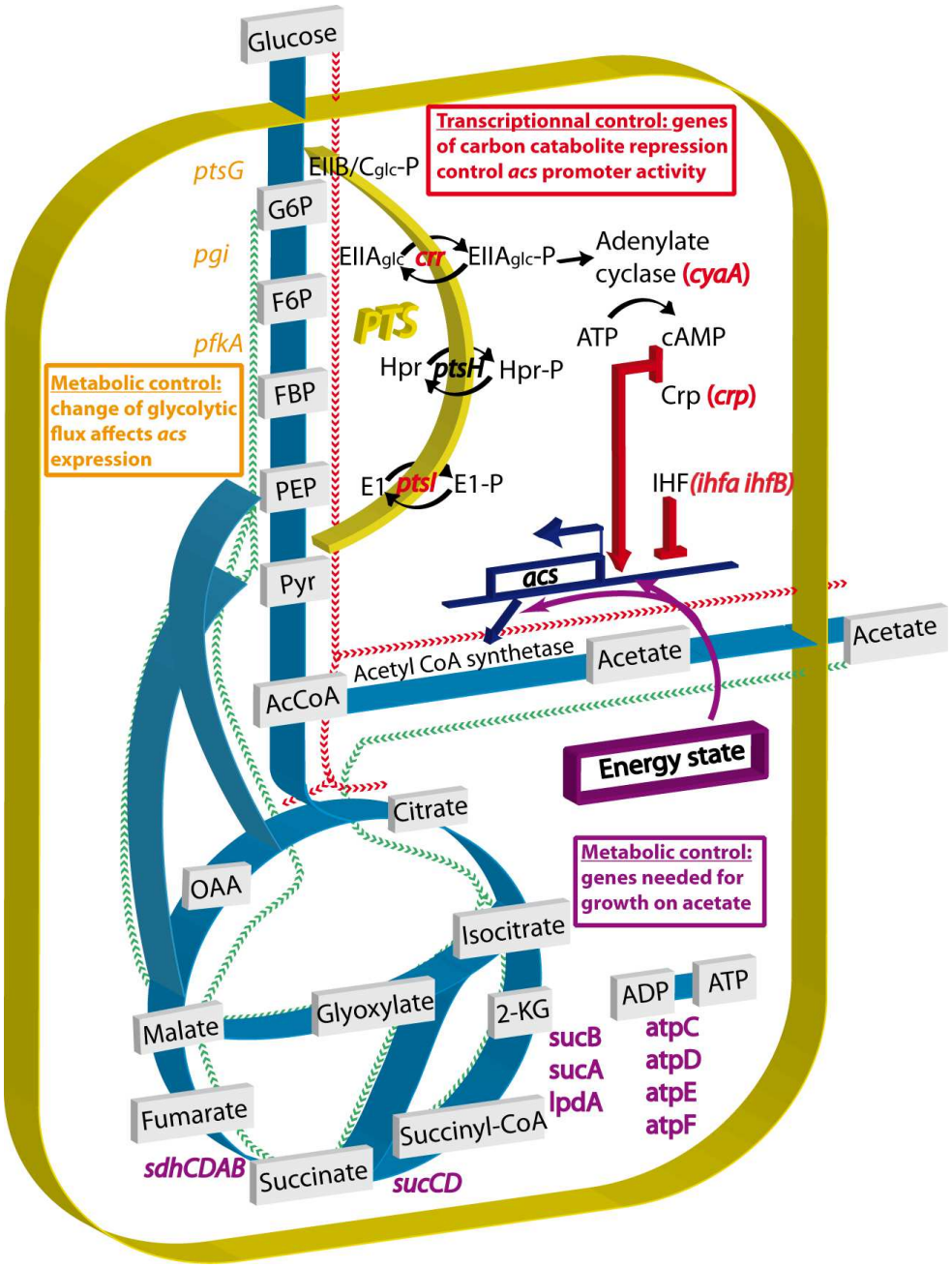

Figure 5. The regulation of *acs* expression. The expression of *acs* is regulated by genes involved in glucose repression (*cyaA*, *crp*, *crr* and *ptsI*). A modification of the glycolytic flux has indirect consequences on *acs* expression (*ptsG*, *pgi*, *pfkA*). Genes of the TCA cycle are needed for acetate utilization and mutants in these enzymes shut down *acs* transcription by a yet unknown mechanism. The red and green routes are schematic representations of the flux direction when cells grow on glucose and acetate, respectively.

our understanding of the precise mechanism that underlie the glucose effect (and more generally the carbon catabolite repression) is still far from complete and remains the subjects of debate (Inada *et al.*, 1996; Crasnier-Mednansky, 2008; Gorke & Stulke, 2008b; Narang, 2009). We are currently exploring theses paradoxical results in more detail.

Metabolism and gene expression

An important strength of our screen is to uncover connections between metabolism and gene expression. In the case of *acs*, we find several novel connections between the metabolic state of the cell and the transcription of *acs*. Although still to be confirmed, it appears that the enzymes involved in the growth on acetate (*suc*, *atp* mutants etc) have a genuine impact on *acs* expression. Even though such a regulation makes sense biologically, the mechanism of this signal transduction remains to be discovered. Another example involves the glycolytic flux. Reducing the glucose inflow by deleting the glucose transporter (*ptsG* gene) increases the basal *acs* expression level during growth on glucose, probably by increasing the intracellular concentration of cAMP. However, the reduction of the glycolytic flux observed during growth of the *pgi* and *pfkA* mutants does not lead to an enhancement of *acs* expression in exponential phase (Fig. 2E). This result shows that neither *acs* promoter activity nor cAMP concentration are a simple function of the glycolytic flux, which is roughly the same for *ptsG*, *pgi*, and *pfkA* strains, but depends on the exact location where glycolysis is interrupted. We do not yet know the metabolic indicator nor the sensing mechanism that transmits the state of glycolysis to the activation of the *acs* promoter.

These different observations clearly show a strong connection between the metabolic state of the cell and *acs* transcription and confirm our prediction that the genetic regulatory network of *Escherichia coli* is densely connected and that a majority of these connections pass through metabolism (Baldazzi *et al.*, 2010).

Materials and Methods

Strains

The screen uses the Keio collection, comprising 3985 single gene deletions of the *E. coli* K-12 strain BW25113, called wild-type strain (Baba *et al.*, 2006).

Plasmids

The nrfA-*acs* intergenic region was amplified by PCR using primers 5'-TCCTCTCGAGAGGGGCTTCA TCCGAAT-3' and 5'-TTTGGGATCCGCTTTTGTTCTCCTTGTAGG-3' and the BW25113 strain as a template. The PCR product, digested with Xho1 and BamHI, was inserted into a *luxCDABE* plasmid backbone without promoter to give p-acs-lux. A strong ribosome binding site drives efficient expression of the reporter gene. The *bla* gene (conferring ampicillin resistance) and the *acs* promoter region of the previous plasmid p-acs-lux were amplified by PCR using primers 5'-TTTGGGATCCGCTTTTGTTCTCCTT GTAGG-3' and 5'-TCTAAAGTGAGCTCGAGTAAACTTGGTCTGACAG-3'. The PCR product, digested with SacI and BamHI, was inserted into pUA66 (Zaslaver *et al.*, 2006) to give p-acs-gfp. The complete sequences of these plasmids are available upon request.

High-throughput transformation

All steps were performed in 96-well plates. 135 plates, 6 days and 4 persons were needed to transform 3571 mutants. All volumes below are indicated for one well. Mutants were inoculated from frozen stock in sterile microplates containing 200 μl per well of Luria-Bertani broth (LB) with kanamycin (50 μg/ml) and placed at 37°C under shaking (200 rpm) overnight. The next day, 4 μl of the pre-culture were added to new plates containing 400 μl of LB with kanamycin (50 μg/ml) and placed at 37°C under shaking for 3 hours. We then transferred 50 μl of these exponentially growing cells to new plates containing 50 μl of ice cold TSS 2X (LB containing 10% PEG, 5% DMSO, and 20-50 mM Mg_{2+} at a final pH of 6.5) (Chung *et al.*, 1989) and the mixture was kept on ice for 45 min before adding 4 μl of plasmid DNA (50 ng/ml). The plates were left on ice for another 10 minutes before applying a heat shock (90 seconds at 42°C) and returned on ice for an additional 10 minutes of incubation. We finally added 1 ml of LB containing ampicillin (100 μg/ml) and the plates were placed at 37°C under shaking overnight. The following day, we spotted the transformed mutants onto two different petri dishes containing either LB-agar-ampicillin (100 μg/ml) or minimal medium M9-agar-0.3% glucose (p/v)-ampicillin (100 μg/ml). Petri dishes were incubated at 37°C overnight and the luminescence of the colonies was measured the following days using an intensified camera (Photonic Science). The quantification of the luminescence of the 3500 colonies of the condition "LB day 1" was performed semi-automatically by means of a custom-made Matlab® scripts (Image processing toolbox). Briefly, we used functions that correct side effects

and compute an appropriate threshold on the images allowing the detection of the colonies. Then, we computed the median of luminescence pixels belonging to a specific colony.

Measuring the dynamics of gene expression

The dynamics of gene expression was measured for cells growing for 10 hours in a 96-well microplate. The microplate reader (Perkin Elmer, Fusion alpha FP-HT) allows growth with shaking and temperature control (37°C). We measured the cell density (OD at 600 nm) every 30 min and luminescence or fluorescence (480 nm/520 nm) every 3 minutes. Unless otherwise specified, the wells in the microplate contain 180 μl of minimal medium (M9) supplemented with 0.03% glucose (w/v), 0.03% sodium acetate (w/v), and ampicillin (100 μg/ml). When indicated, we added cyclic AMP (5 mM) at the beginning of the experiment. The wells were inoculated with 20 μl of stationary phase cultures (10x dilution) grown in LB-ampicillin (100 μg/ml). The fluorescence background of the bacterial cells was assessed by measuring the fluorescence of mutant bacteria transformed with a promoterless reporter plasmid.

Quantification of the expression profiles

In order to compare the strength of *acs* induction at the entry into stationary phase as measured by the luminescence and fluorescence reporter systems, we have calculated the area under the expression profile for four hours following entry into stationary phase (see Figure 4B for a schematic representation). This quantification was carried out using custom-made Matlab® scripts that perform the following tasks: (i) Remove outliers from the data. (ii) Determine the time of entry into stationary phase by classifying the expression profiles into different classes and fitting segment splines to the signal (fluorescence or luminescence) and the absorbance measurements. The entry into stationary phase is, e.g., indicated by a break in the absorbance curve. (iii) Correct the fluorescence expression profiles for the autofluorescence of the cells by subtracting corresponding profiles of control strains. No background correction is necessary for luminescence data. (iv) Numerically integrate the expression curve for 240 min, starting at the entry into stationary phase. The expression value was set to zero at the entry into stationary phase and the integral thus gives a measure of the induction strength of the *acs* promoter in the particular mutant strain. (v) Normalize the integral by the absorbance of the sample at entry into stationary phase.

qRT-PCR

The strains were grown in a microplate containing M9 0.03% glucose and 0.03% acetate (preculture LB). The cells (10^8) were harvested either before glucose exhaustion (exponential growth on glucose) or after glucose exhaustion (growth on acetate). Total mRNA was protected using the RNAprotect® Bacteria Reagent (Quiagen) and then isolated using the RNeasy mini kit (Quiagen) according to the recommendations of the manufacturer. Potential trace quantities of DNA were removed using the turbo DNAse (Ambion). Samples were protected from RNase using RNaseOut ribonuclease inhibitor (Invitrogen). 1 μg of RNA was reverse transcribed using SuperScript^{TM} II Reverse Transcriptase (Invitrogen) according to the protocol of the manufacturer. Briefly, a 25 μl reaction mixture was incubated in a T3000 thermocycler (Biometra) for 10 min at 25°C, 50 min at 42°C and 15 min at 70°C. The primers (Eurogentec) used in the present study were as follows: *acs*: 5' ACAGTTCTGGTGACGGTTCC 3' and 5' ACAGTTCTGGTGACGGTTCC 3'. We have used the *rrsD* gene as internal control for relative quantification: *rrsD*: 5' GCTACAATGGCGCATACAAA-3' and 5'-TTCATGGAGTCGAGTTGCAG 3'. Quantitative PCR was performed in a StepOnePlus Real-Time PCR System (Applied Biosystems) using MESA Green qPCR Master Mix (Eurogentec) according to the instructions of the manufacturer. Briefly, 25 μl reactions mixtures were incubated for 10 min at 95°C and 40 PCR cycles (15 s at 95°C and 1 min at 60°C). PCRs were run in triplicate. Raw data were transformed into threshold cycle (Ct) values. The relative strength of *acs* expression for each mutant, compared to wild-type, was calculated by the comparative Ct Method ($\Delta\Delta$Ct).

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Supplementary information

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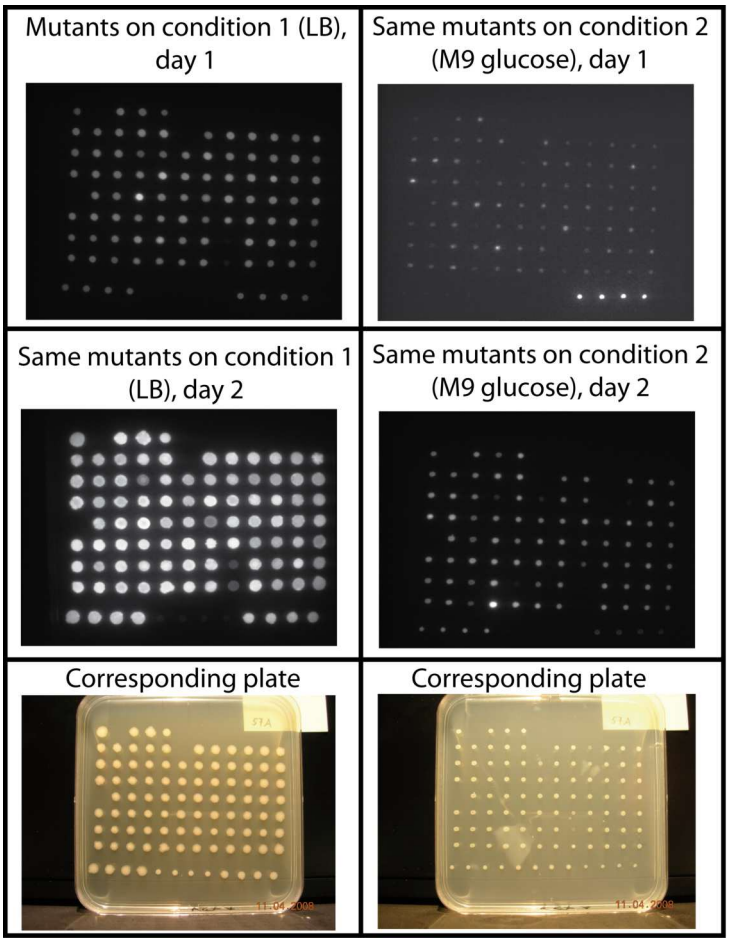
1 The screen can be performed in different conditions



The pattern of luminescence of colonies can significantly change over time and as a function of the growth medium. Our screen allows to easily test different conditions and to perform kinetics at the colony level. Here we show a typical example of a screen where the cells were grown on LB (left column) or M9-glucose minimal medium (right column). The luminescence images were taken after one or two days of incubation. The growth of the colonies is shown in the bottom row. The supplementary data show the luminescence images of colonies grown on LB plates. Luminescence images of M9 plates (and/or colony images) are available upon request.

2 The screen can be performed dynamically

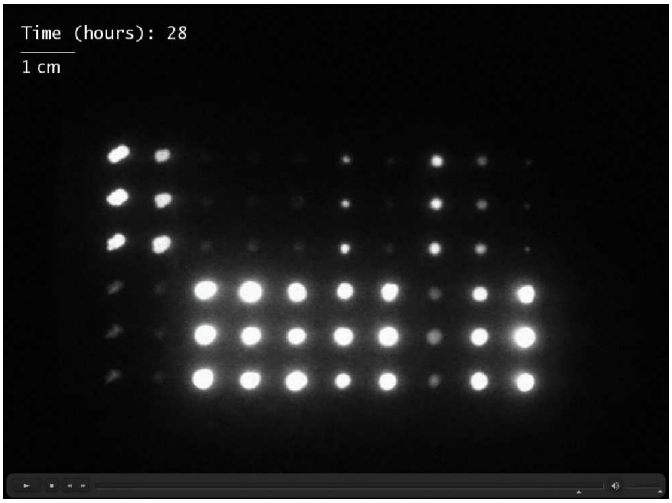


Figure 1. Video of the development of colonies over time

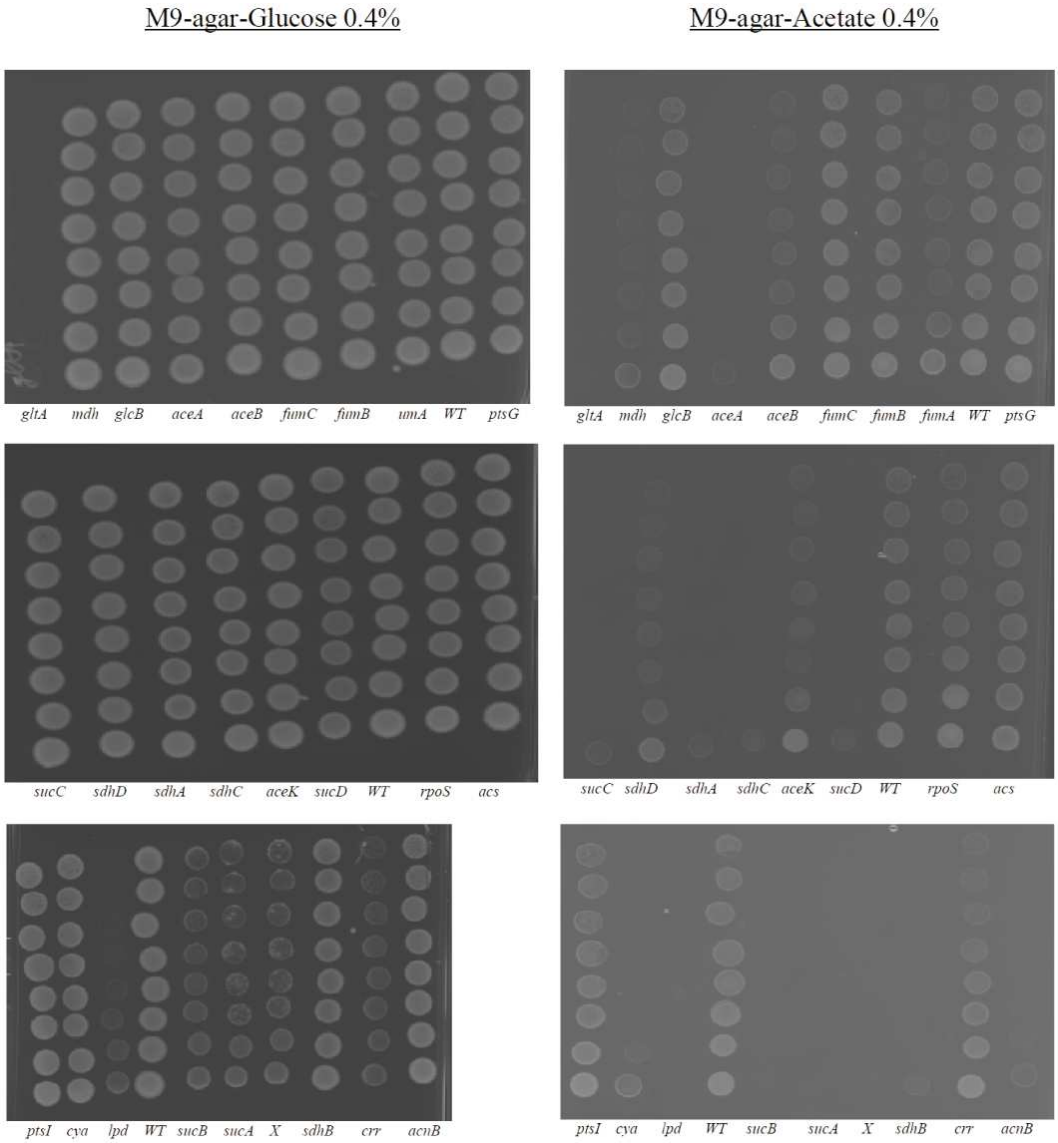
Wt	Wt 2	cyaA-	crp-	ptsI-	ptsH-	crr-	ptsG-	pgi-	pfkA-
Wt	Wt 2	cyaA-	crp-	ptsI-	ptsH-	crr-	ptsG-	pgi-	pfkA-
Wt	Wt 2	cyaA-	crp-	ptsI-	ptsH-	crr-	ptsG-	pgi-	pfkA-
sucB-	lpdA-	ihfA-	ihfB-	fis-	iclR-	fnr-	cpdA-	rpos-	hha-
sucB-	lpdA-	ihfA-	ihfB-	fis-	iclR-	fnr-	cpdA-	rpos-	hha-
sucB-	lpdA-	ihfA-	ihfB-	fis-	iclR-	fnr-	cpdA-	rpos-	hha-

Mutants (see the table) containing the p-acs-lux plasmid were "spotted" on solid M9 medium 0.06% glucose 0.06% acetate 10% LB 1% agar 100 μg/ml ampicillin. We have filmed the development of colonies (luminescence) for 30 hours using an intensified camera.

Results

- The expression profiles of triplicate colonies show identical and highly reproducible kinetics.
- The initial Keio *crp* strain we used in the screen was incorrectly constructed. A correct copy of this strain shows the same phenotype than the *cyaA* strain, as expected.
- Wt 2 is a strain with a shorter *acs* promoter sequence that does not include the *acsP1* promoter (maintaining only the *acsP2* promoter). Similar expression patterns are obtained, except that the intensity of *acs* expression is somewhat smaller.

3 Mutants that do not grow on minimal medium acetate



Certain mutants that grow on M9+Agar+glucose (0.4%) do not grow on M9+agar+acetate (0.4%). The y axis (upward) represents serial dilutions (factor 10) of strains pre-cultured in LB medium.

4 Mutants that do not grow on minimal medium glucose

Mutants that grow on Luria Broth + Agar + ampicillin but do not grow on M9 + agar + 0.3% glucose + ampicillin

ppc	icd	rpoN	gltA	hisH	argC	ycaL	argG	cysG	trpB	metB	metR
proB	trpD	glyA	serA	argE	thrB	proA	serC	trpE	argH	aroA	hisG
pheA	metA	leuD	aroD	hisD	aroC	tyrA	aroE	ilvE	leuC	proC	hisC
ilvA	leuA	hisF	argA	metE	leuL	serB	nadB	pdxA	nadC	nadA	pdxJ
metF	cysN	cysC	cysD	cysH	purK	carB	purE	pyrD	pyrC	aroB	pyrE
pyrB	pyrF	purD	purA	purM	fepD	fepB	purL	hisB	hisA	trpC	argB
thrC	ilvC	ilvD	thrA	purH	purC	cysB	guaB				

Mutants with strong growth defects on M9+agar+0.3% glucose+ampicillin

sucA	sucB	lysR	Rpe	pfkA	Pgi	Rnt	aceE	cydB	Lpd	ybaS	fepC
metC	bioF	pabB	pabA	bioC	bioD	panC	lipA	panB	Cmk	pgpA	cysQ
ptsH	ptsI	gcvR	metL	fepG	carA						

Chapitre III

Study of carbon catabolite repression in *Escherichia coli* by monitoring the activity of Crp-cAMP dependent promoters

Study of carbon catabolite repression in *Escherichia coli* by monitoring the activity of Crp-cAMP dependent promoters

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Abstract

Despite decades of intensive study, the understanding of the precise mechanism underlying carbon catabolite repression (CCR) in *Escherichia coli* is still far from complete. The central regulator of CCR is Crp, which becomes active in the presence of cAMP. However, addition of exogenous cAMP often fails to elicit the expected response. In this study, we have investigated some of the reasons for this unexpected result. We measured the activity of numerous CRP-cAMP dependent promoters in different conditions and genetic backgrounds. We used two different reporter genes (GFP and luciferase), measuring expression kinetics at the population level and at the single cell level. Our results indicate that the mechanism of carbon catabolite repression involves a metabolic control unrelated to the concentration of cAMP or to the phosphate transfer system.

Introduction

In microorganisms, certain carbon sources are preferred to others. For example, when the medium contains glucose and lactose, *Escherichia coli* preferentially uses glucose and starts to metabolize lactose only when glucose is exhausted (Monod, 1942). This phenotype is explained by the inhibitory effect of glucose (the so-called glucose effect) on the expression of genes involved in lactose catabolism: the extensively studied *lac* operon. The phenomenon has been observed with other carbohydrates in many different prokaryotes and has been called carbon catabolite repression (CCR) (Magasanik, 1961). It refers to the general phenomenon whereby the presence of certain carbon sources in the medium represses the expression of certain genes or operons (Gorke & Stulke, 2008a).

Two complementary mechanisms have been proposed to explain the glucose mediated catabolite repression in *E. coli*: the "inducer exclusion model" (Saier & Roseman, 1972) and the "cAMP model" (Ullmann & Monod, 1968; Perlman *et al.*, 1969). The uptake of glucose is mediated by the phosphopenolpyruvate:glucose phosphotransferase system (PTS). This system sequentially transfers a phosphoryl group originating from phospoenolpyruvate to glucose via several enzymes including $EIIA_{glc}$ (Postma *et al.*, 1993). The cornerstone common to both models is the phosphorylation state of the enzyme $EIIA_{glc}$.

In the inducer exclusion model, the dephosphorylated form of $EIIA_{glc}$, the dominant form in the presence of glucose, binds and thereby inhibits different sugar transport systems, thus preventing the entry of the inducing sugar into the cell.

In the cAMP model (Kolb *et al.*, 1993), the phosphorylated form of $EIIA_{glc}$, the dominant form in the absence of glucose, binds and thereby activates adenylate cyclase, the enzyme that converts ATP into cAMP. The concentration of cAMP quickly increases, binds to Crp (cAMP receptor protein) and converts this global transcription factor to the active form, Crp-cAMP. Figure 1 summarizes these two models, taking as an example the well-studied regulation of *lacZ* expression.

Yet, despite more than four decades of research, our understanding of the precise mechanism undelying carbon catabolite repression remains incomplete and subject to debate (Inada *et al.*, 1996; Crasnier-Mednansky, 2008; Gorke & Stulke, 2008b; Narang, 2009a). Although the two models have a certain predictive power, each one has to struggle to account for experiments contradicting particular model predictions. For instance, glucose repression have still been observed in a *crp** strain which harbors a modified Crp protein that binds to the Crp-cAMP binding site even in absence of cAMP (Inada *et al.*, 1996; Khankal *et al.*, 2009). Moreover, both models fail to explain why PTS-independent carbon sources, such as glucose-6-phosphate, induce a strong catabolite repression even in PTS-deficient strains (Hogema *et al.*, 1997).

In a previous study (article not yet published), we studied the expression of *acs*, a gene whose transcription is strongly repressed by glucose and positively regulated by the CRP-cAMP complex (Kumari *et al.*, 2000). We described several experiments whose results did not fit well with the current models of carbon catabolite repression. In particular, we failed to relieve glucose repression by adding exogenous cAMP to the growth medium. Here, we investigate in detail the reasons for these unexpected results. We first measured the *acs* promoter activity in different conditions and in different genetic backgrounds by using two different reporter genes (GFP and luciferase). We then extended our investigation to other

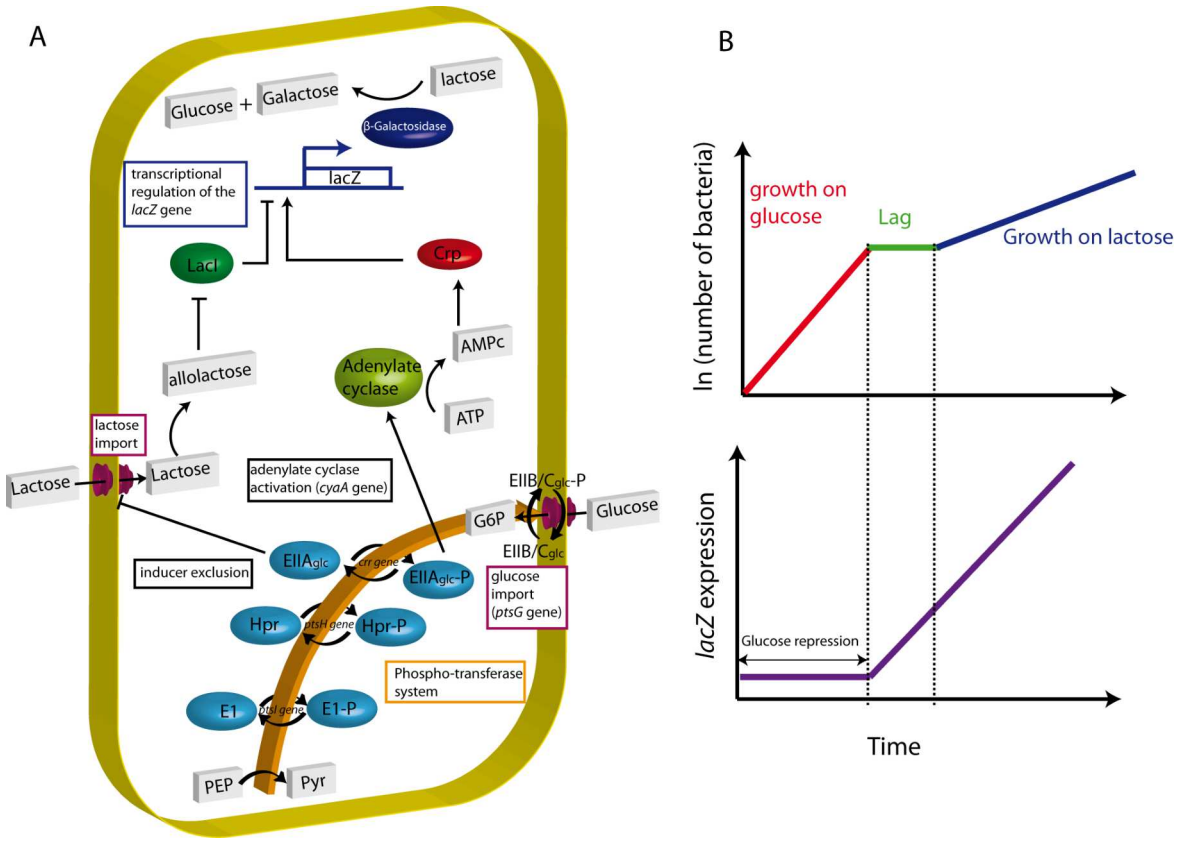

Figure 1. The two models explaining glucose repression of the *lac* operon. (A) Glucose import is mediated by the PTS system, which transfers a phosphoryl group originating from PEP to glucose. The inducer exclusion model: during glucose import, the dephosphorylated form of $EIIA_{glc}$ inactivates the import of lactose. This prevents the inactivation of the LacI repressor. Consequently, *lacZ* transcription is repressed. The cAMP model: once glucose is exhausted, the phosphorylated form of $EIIA_{glc}$ becomes predominant. It activates adenylate cyclase, which produces cAMP. This signalling molecule binds to the Crp protein, and the resulting complex activates *lacZ* transcription. (B) Schematic representation: the absence of β-galactosidase during growth on glucose leads to diauxic growth where lactose is consumed only after glucose exhaustion.

Crp-cAMP dependent promoters. Our results show that the mechanism of carbon catabolite repression cannot be totally explained neither by low levels of intracellular cAMP, nor by the phosphorylation state of $EIIA_{glc}$. We conclude that a reduction of the glycolytic flux, unrelated to the PTS or cAMP level and detected by an as yet unidentified metabolic sensor, is needed to fully induce Crp-cAMP dependent promoters.

Results

The *acs* promoter is strongly controlled by carbon catabolite repression

To measure *acs* expression, we used the previously described transcriptional fusions p-acs-lux and p-acs-gfp (article not yet published). Briefly, the *acs* promoter is cloned either upstream *luxCDABE* or *gfpmut2* reporter genes on a plasmid carrying either the colE1 (p-acs-lux) or the pSC101 (p-acs-gfp) origin of replication. To measure gene expression, we grow cells on minimal medium with the indicated carbon sources for several hours within a thermostated micro plate reader with periodic agitation. We measured absorbance, luminescence and fluorescence at intervals of about 5 minutes.

In a wild-type *Escherichia coli* strain growing on minimal medium (M9) supplemented with 0.03% glucose and 0.03% acetate, we observe a strong induction of *acs* expression when growth slows dramatically (glucose exhaustion) (Figure 2A). For reasons of clarity, we will not show the growth curves in the following profiles. However, to assist readers, we generally indicate the time of glucose exhaustion by an arrow. Identical profiles are obtained using the two reporter systems and plasmids carrying two different origins of replication. In addition, we have verified the expression profile by an independent method, qRT-PCR (article not yet published). We can therefore ascertain that the signal we measure reflects transcriptional regulation of the *acs* gene.

We observe an essentially identical expression profile when growing the wild-type strain on the non-PTS sugar glucose-6-phosphate (Figure 2B). This experiment demonstrates that the *acs* promoter is also strongly repressed by glucose-6-phosphate. Adding exogenous cAMP (5 mM) at the beginning of the experiment fails to directly induce the expression of the *acs* gene (Figure 2C). Adding glucose to the well during the experiment after glucose exhaustion, i.e., during the induction of *acs*, immediately stops the *acs* promoter activity. A *acs* induction occurs again when the newly added glucose is exhausted. The more glucose was added, the longer the duration of the shutoff of *acs* (Figure 2D). The time it takes for

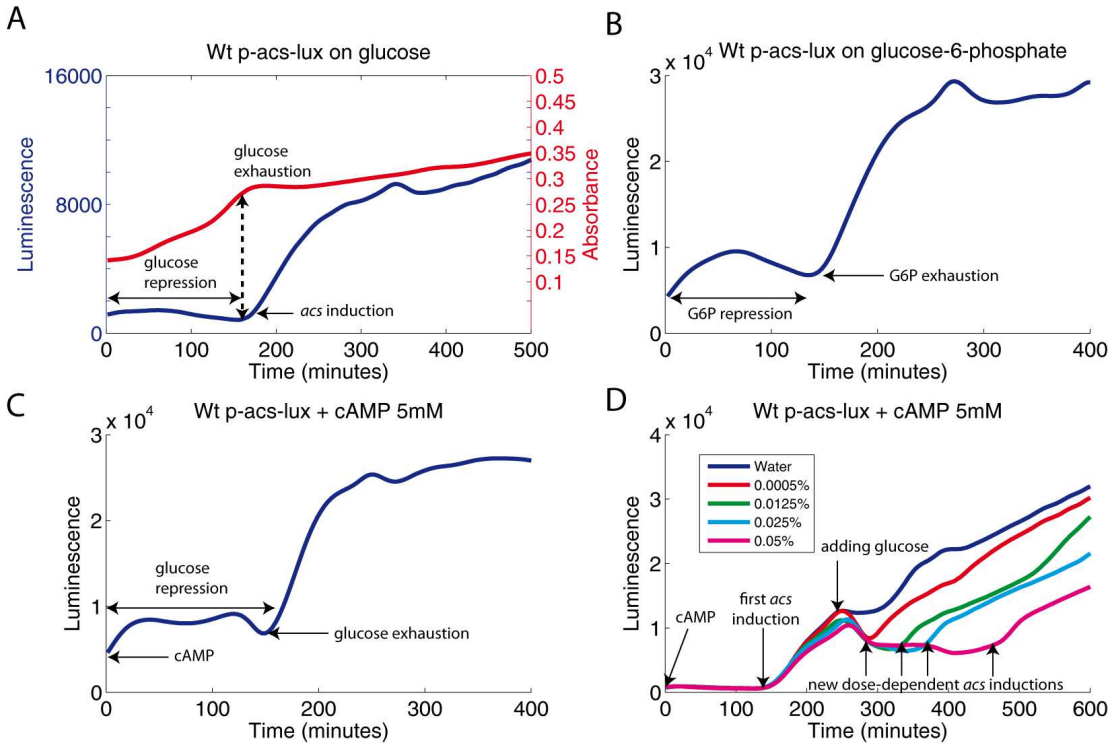

Figure 2. *acs* expression in the wild-type strain. (A) *acs* expression measured by the luciferase reporter in the wild-type strain growing on minimal medium, supplemented with 0.03% glucose and 0.03% acetate. The expression of *acs* is inhibited by a strong glucose repression and is induced once glucose is exhausted. (B) A strong repression also occurs in the presence of a non-PTS sugar (glucose-6-phosphate). (C) Glucose repression persists even when cAMP (5mM) is added to the medium at the beginning of the experiment. (D) *acs* induction is interrupted by adding glucose to the medium. A new induction occurs when this newly added glucose is exhausted (in a dose-dependent manner). This switch, controlled by glucose, occurs despite the presence of exogenous cAMP.

the expression of *acs* to stop when adding glucose (well below 10 minutes) is not compatible with the transcription and translation of a new hypothetic regulator. Thus, the repression occurs at the time-scale of signal transduction, e.g., the sensing of a biochemical signal. This switching behavior triggered by glucose is independent of the variation of the cAMP concentration, since cAMP is added in excess to the medium at the beginning of the experiment.

Role of Crp-cAMP in carbon catabolite repression of the *acs* promoter

To clearly define the roles of Crp and cAMP in the control of *acs* expression , we measured the *acs* promoter activity in an adenylate cyclase deficient strain (*cyaA* mutant) and in a *crp* mutant. In both strains, no transcription of *acs* occurs, demonstrating the total dependence on the Crp-cAMP complex (controls on Figure 3A and 3B). In the *cyaA* strain, adding exogenous cAMP at the beginning of the kinetics restores the same *acs* transcription profile as the one observed in a wild-type strain (Figure 3A), showing strong *acs* induction only when glucose is exhausted. The lack of *acs* expression during growth on glucose could be due to the absence of Crp during this growth phase. To exclude this possibility, we constructed a plasmid that over-express the Crp protein when induced by anhydrotetracycline (aTc). This plasmid complements the *crp* mutant in a dose dependent manner (aTc), but only when glucose is exhausted (Figure 3B).

The sudden induction of *acs* in the wild-type strain and its absence in a *cyaA* strain have also been observed at the single cell level using time-lapse microscopy. We grew these two strains (transformed with the p-acs-gfp plasmid) together on a solid and translucent medium and acquired fluorescence and phase contrast images for several hours. As expected, only part of the bacterial layer (corresponding to the wild-type strain) showed a sudden increase of fluorescence once the micro colony has stopped growing (Figure 3C; supplementary movie 1). Furthermore, in this experiment, the growth conditions (oxygenation, growth rate, contact with neighboring bacteria) are quite different from the conditions of growth in a homogeneous, liquid culture. Yet, the observed expression profile is remarkably resilent.

Role of the PTS in carbon catabolite repression of the *acs* promoter

To assess the role of the PTS in glucose-mediated repression of the *acs* promoter, we had previously measured its activity in two PTS mutants: the *ptsI* and *crr* strains. The E1 deficient strain (*ptsI* mutant) has a much lower growth rate on glucose. Since this mutant completely inactivates the PTS by blocking

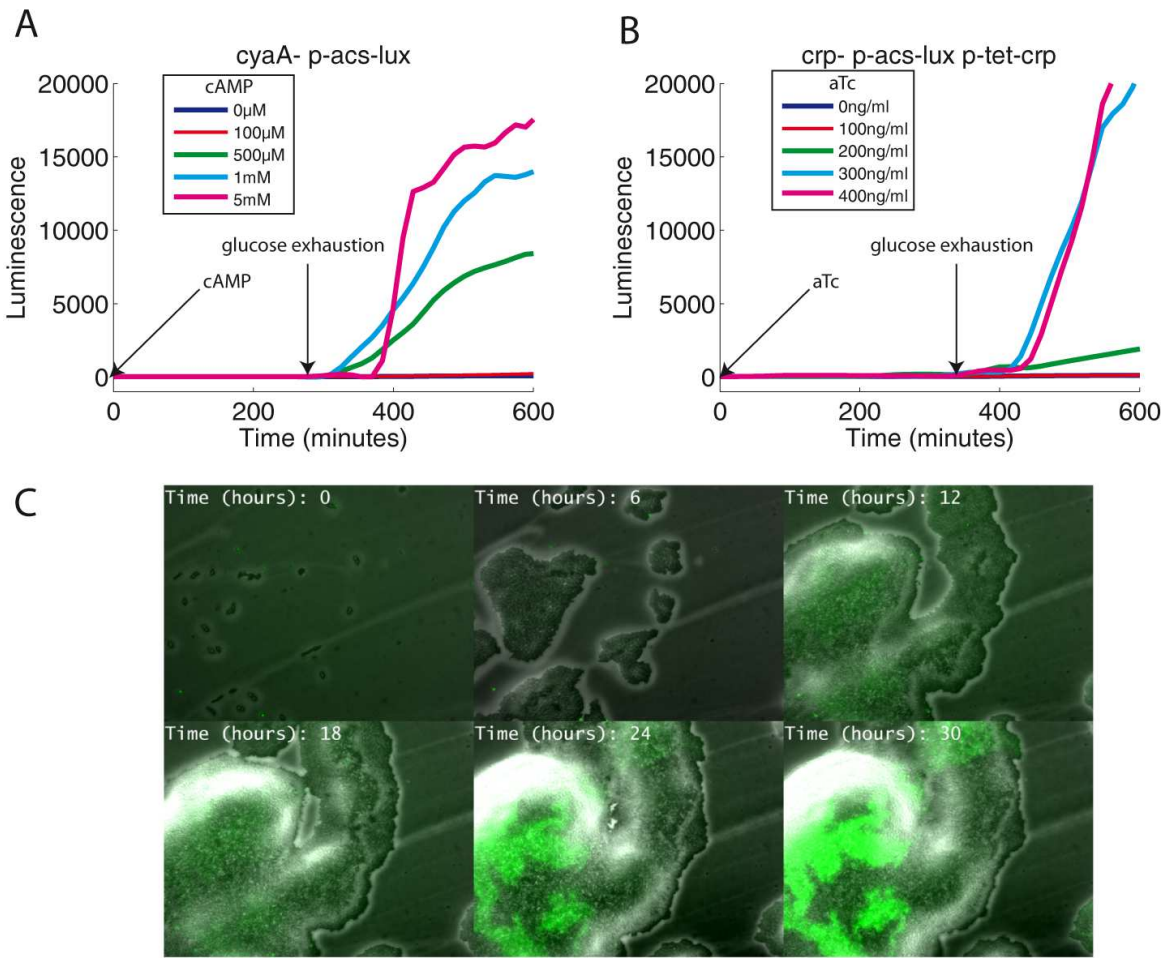

Figure 3. *acs* expression in *cyaA* and *crp* mutants. (A and B) Bacteria were grown on a minimal medium supplemented with glucose and acetate. The controls (dark blue) show that the absence of *crp* or *cyaA* prevents the expression of *acs*. (A) The *cyaA* strain is complemented in a dose-dependent manner by adding cAMP to the medium. (B) Similar dose-dependent complementation is observed with the *crp* mutant, by using a plasmid (p-tet-crp) allowing anhydrotetracycline (aTc) dependent expression of Crp. (A and B) In both cases, the repression remains while glucose is still present. (C) Time-lapse microscopy. A wild-type strain and a *cyaA* mutant mixed together were grown on the surface of a solid medium. After growth arrest (glucose exhaustion), only a part of the bacterial layer (corresponding to the wild-type strain) induces *acs* expression, as shown by the rise in fluorescence.

the first step of phosphate transfer, glucose has to enter the cell via PTS-independent transporters (MglC and GalP) (Gosset, 2005). *acs* expression in this strain is very weak, suggesting the role of the PTS in the production of cAMP. Adding exogenous cAMP restores *acs* transcription, even in presence of glucose (Figure 4A).

Restoring a fast growth rate by providing the favorable, non-PTS sugar glucose-6-phosphate re-established the repression of *acs* transcription in presence of exogenous cAMP (Figure 4B). First, this experiment shows that glucose-6-phosphate maintains a strong repression of the *acs* promoter, even in a PTS-deficient strain. Therefore, the repression of the *acs* promoter cannot be due to a modification of the PTS phosphorylation state by glucose-6-phosphate. Second, the major difference between growth on glucose and growth on glucose-6-phosphate in a *ptsI* mutant, and in the presence of exogenous cAMP, is the growth rate. The *ptsI* strain has a strong growth defect on glucose whereas it grows normally on glucose-6-phospate which is transported by a PTS independent hexose-phosphate system. Once in the cell, both substrates are metabolized by glycolysis. The *acs* promoter thus seems to somehow sense the rate of glucose metabolism and is activated when glycolysis is slow.

We also measured *acs* expression in an $EIIA_{glc}$ deficient strain (*crr* mutant), i.e. a strain that lacks the component of the PTS at the crossroad between inducer exclusion and activation of adenylate cyclase. The *crr* mutant grows at an almost normal rate on glucose, i.e., much faster than the *ptsI* strain. Since there are several alternative EIIA proteins that can participate in the PTS, the absence of $EIIA_{glc}$ may probably be compensated by another EIIA protein. The *acs* promoter activity in this strain is very weak (Figure 4C). Exactly as observed with the *cyaA* mutant, exogenous cAMP restores *acs* expression with a profile very similar to the wild-type pattern. These observations suggests that $EIIA_{glc}$ is important for stimulating adenylate cyclase when glucose is absent from the growth medium. However, this experiment also points out an important shortcoming of current models of CCR: the simultaneous absence of a $EIIA_{glc}$ mediated hypothetical (acetate has never been described as an inducer of *acs* expression) inducer exclusion and the presence of exogenous cAMP are not sufficient to remove glucose repression. This result is further confirmed in the *crr cyaA* double mutant (Figure 4D). Glucose repression is clearly maintained until all glucose is exhausted. Thus, deleting the two main components of carbon catabolite repression has failed to remove the glucose mediated *acs* repression. Neither the "cAMP model" nor the "inducer exclusion model" are able to explain this result. Potential explanations involving a major role of Fis, IHF, IclR, FNR, RpoS, FruR or Mlc were discarded by using the corresponding mutants (data not shown).

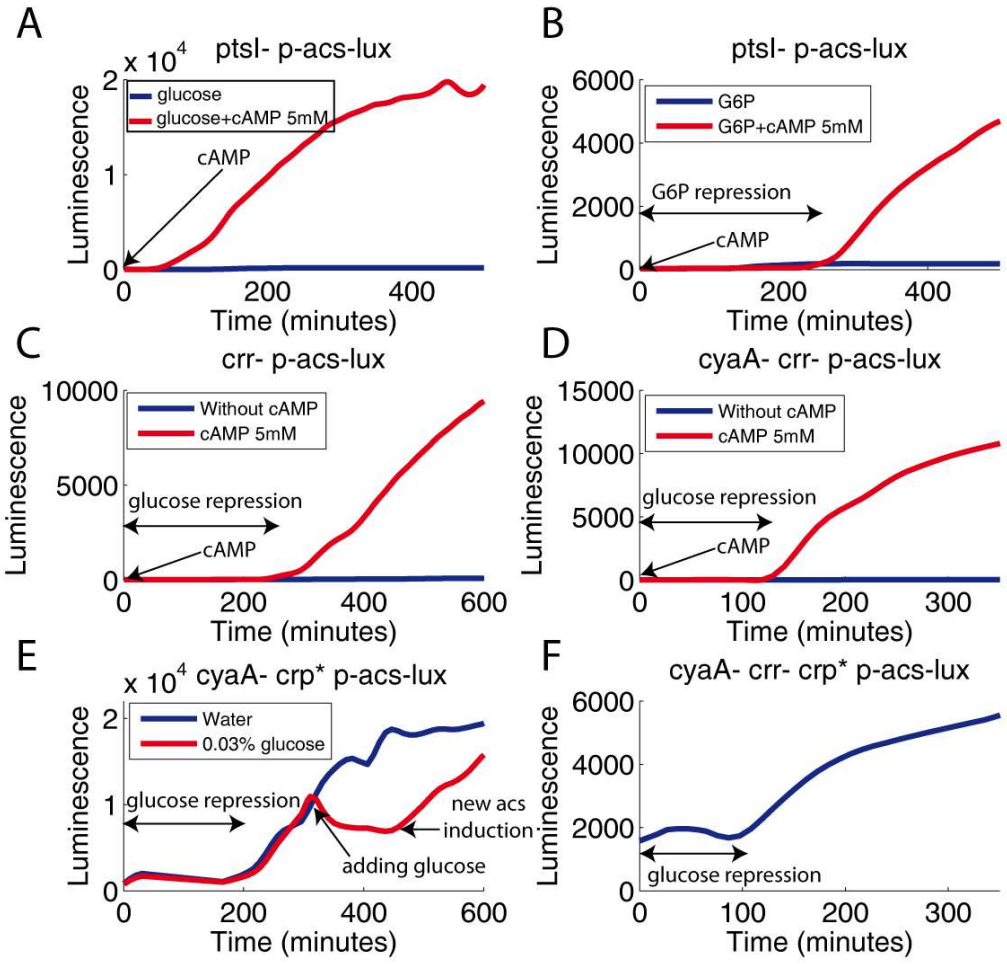

Figure 4. *acs* expression in PTS deficient strains and *crp strains.** Bacteria were grown on a minimal medium supplemented with 0.03% glucose and 0.03% acetate. (A and C) *ptsI* and *crr* strains have an extremely low *acs* expression (control in dark blue). (A) cAMP (5mM) complements the *ptsI* strain even during growth on glucose. (B) cAMP complements the *ptsI* strain, but only when G6P (0.03%) is exhausted. (C and D) Glucose repression remains in *crr* and *crr cyaA* strains, even when cAMP is added to the medium. (E) The *acs* expression is recovered in a *cyaA crp** strain without the need of adding cAMP. Yet, the glucose repression is still observed. Adding glucose just after *acs* induction stops the *acs* promoter activity and a new induction occurs when the added glucose is exhausted. (F) The glucose repression is still observed in a *cyaA crr crp** double mutant.

Role of cAMP import/export in carbon catabolite repression of the *acs* promoter

One possible explanation of the previous results is that glucose controls the import/export of cAMP and thus prevents the entrance of exogenous cAMP (Buettner *et al.*, 1973). Indeed, the mechanism of import/export of cAMP remains largely unexplained, even though recent results suggest that TolC is partly responsible for the active export of cAMP (Hantke *et al.*, 2011). To assess a possible control of cAMP import/export as an explanation of our results, we constructed a *cyaA crp** strain harboring a modified Crp* protein carrying three amino acid substitutions (I112L, T127I, and A144T) (Khankal *et al.*, 2009). This Crp* protein binds to the Crp-cAMP DNA binding site even in the absence of cAMP. We confirm that the three amino acid difference between the *cyaA* mutant and the *cyaA crp** strain is sufficient to restore *acs* transcription without the need of adding exogenous cAMP to the medium. However, again, induction of the *acs* promoter occurs only after glucose exhaustion. Furthermore, adding glucose just after the first *acs* induction immediately stops *acs* promoter activity. A new induction occurs when the newly added glucose is exhausted (Figure 4E). This experiment excludes the possibility that the control of cAMP export or import be responsible for the glucose repression. We obtained similar results using a *crr cyaA crp** triple mutant (Figure 4F). In other words, we observe a strong glucose repression in a strain without cAMP nor $EIIA_{glc}$.

Carbon catabolite repression of other Crp-cAMP dependent promoters

Are our results a particular quirk of the *acs* promoter or a general characteristic of CCR? In order to answer this question, we studied the expression dynamics of five other Crp-cAMP dependent genes: *araBAD*, *glpA*, *sdhC*, *gntT*, *uhpT*. All corresponding transcriptional fusions to *gfp* (Zaslaver *et al.*, 2006) are clearly sensitive to exogenous cAMP. With the exception of *sdh*, these promoters also need inducers to be activated: arabinose (*araBAD*), glycerol (*glpA*), glucose-6-phosphate (*uhpT*) and gluconate (*gntT*) were therefore added as needed.

For the *sdh* promoter, we also constructed the corresponding transcriptional fusion to the luciferase operon. To minimize the probability of any artifact due to the plasmids or reporter systems, we transformed the *cyaA* mutant with both plasmids (p-sdh-lux and p-sdh-gfp) in order to simultaneously monitor luminescence and fluorescence signals (Figure 5A). Figures 5B and 5C confirm that the expression profile

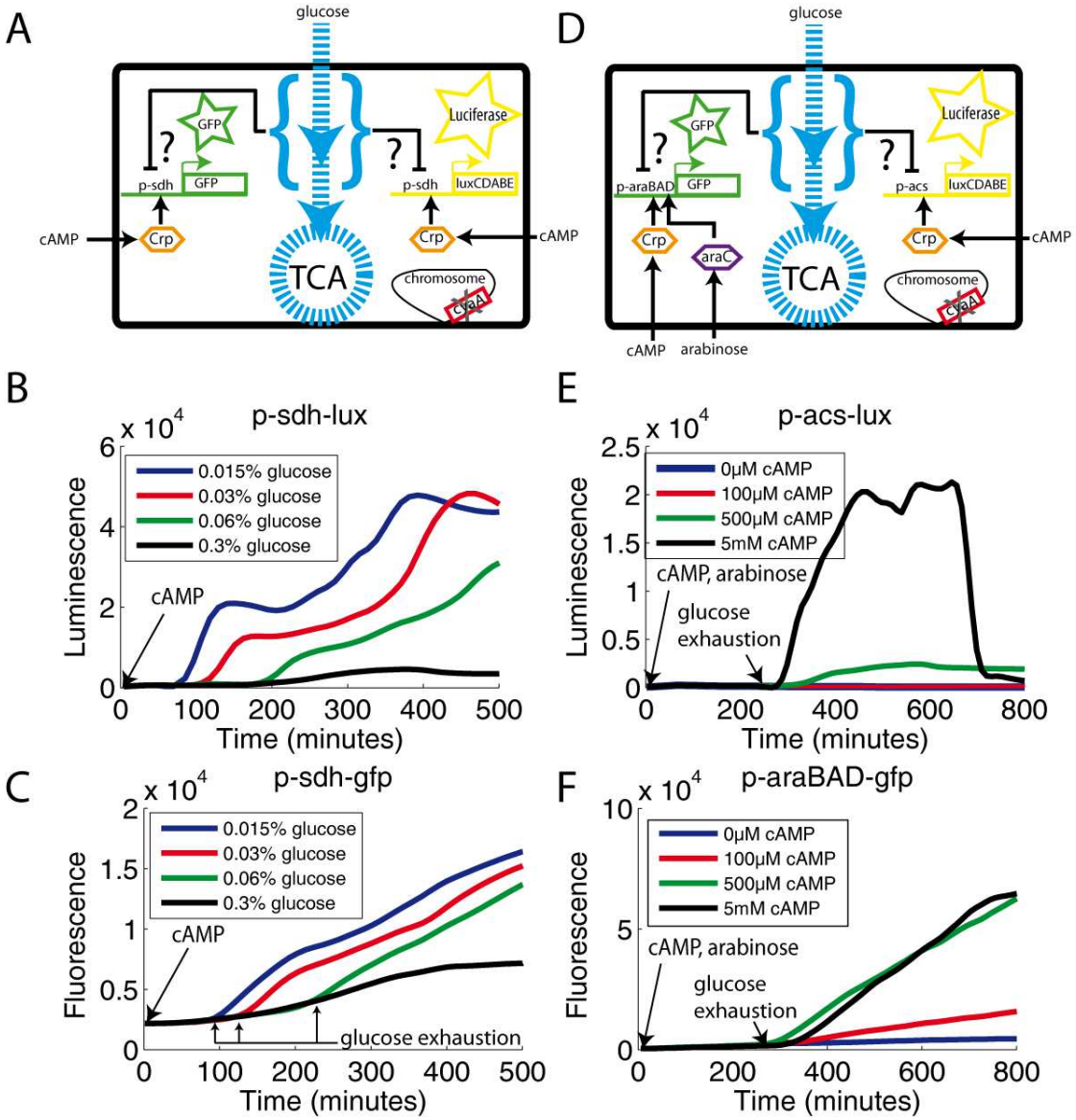

Figure 5. Other cAMP-Crp promoters. Bacteria were grown on a minimal medium supplemented with glucose (as indicated or 0.03%) and 0.03% acetate. (A) The *cyaA* mutant used to obtain the data of figures 5B and 5C. Both GFP and luciferase report *sdh* expression. (B and C) The glucose repression remains, despite the presence of exogenous cAMP (500μM). Increasing concentrations of glucose lengthens the period of growth and thus the period of corresponding repression. (D) The *cyaA* mutant used to obtain the data of figures 5E and 5F. GFP reports *araBAD* expression and luciferase reports *acs* expression. (E and F) The glucose repression remains, despite the presence of cAMP and arabinose (0.01%).

is independent of the reporter system, even though GFP is somewhat less reactive than luciferase. This is expected, since GFP has to maturated the fluorophore before emitting a signal, whereas luciferase is active as soon as the protein is synthesized. In this experiment, we added 500µM cAMP to the medium at the beginning of the experiment. As observed with the *acs* promoter, the *sdh* promoter is fully activated only after glucose exhaustion. Increasing concentrations of glucose extend the duration of the growth phase and correspondingly glucose repression (Figure 5B and C).

We also used the double reporter technique to simultaneously monitor the activity of two different promoters (*araBAD*) and (*acs*) in the same cell (Figure 5D). In this experiment, we grew the *cyaA* mutant on glucose and acetate (the carbon sources), in the presence of arabinose and cAMP (the inducers). Both luminescence (*acs*) and fluorescence (*araBAD*) strongly increase after glucose exhaustion (Figures 5E and 5F). As expected, the promoter strength after glucose exhaustion is a function of the cAMP concentration, *araBAD* reaching maximal activity at a lower cAMP concentration than *acs*. Thus, the *sdh* and *araBAD* promoters behave as the *acs* promoter: full induction occurs only after glucose exhaustion, even when cAMP is present in the medium. The same holds true for the *glpA* promoter (see supplementary information). The behavior of the *acs* promoter is therefore representative of several CCR-controlled promoters. Furthermore, for these promoters, the induction behavior mirrors the one of *acs* also in the "cAMP and $EIIA_{glc}$ free" strain (*cyaA crr crp**) (supplementary information). However, we failed to observe glucose repression for the *uhpT* and *gntT* promoters even though the strength of their promoter activity was easily controlled by ranging cAMP (see discussion and supplementary information).

Role of the glycolytic flux

The experiments shown in figure 4 show that neither the functioning of the PTS nor the variation of cAMP concentration explain carbon catabolite repression. The major, systematic difference between situations where the *acs* promoter is induced or not is the growth rate, and more specifically the glycolytic flux (the rate of glycolysis, going from glucose-6-phosphate to pyruvate). For example, glucose and glucose-6-phosphate, after having entered the cell are both in the form of glucose-6-phosphate. The difference in our experiments is the rate of import of these molecules in different situations and mutant backgrounds. In agreement with this hypothesis, we had previously observed, that *acs* expression in a *ptsG* strain (deleted for the main glucose transporter) was clearly upregulated compared to the wild-type strain during growth on glucose (article not yet published). The *ptsG* strain is able to grow on glucose by using

other transporters, but its growth rate, and thus its glycolytic flux, is strongly reduced.

We therefore set out to verify a prediction of this hypothesis: we should be able to control carbon catabolite repression by modifying the glycolytic flux. This can be done by supplementing the growth medium with a non metabolizable analogue of glucose, α-methyl glucose. We could indeed induce several Crp-cAMP operons by adding the appropriate concentration of α-methyl glucose into the growth medium. An example is shown in figure 6A, where we control the activity of the *sdh* promoter in a *cyaA* mutant (supplemented with exogenous cAMP) by increasing the concentration of α-methyl glucose. Thus, we do not observe catabolite repression induced by non metabolizable sugars (Ambrose & MacPhee, 1998).

We performed a similar experiment at the single cell level. We filmed 4 different wells in parallel (6-wells microplate). Each well contains a solid medium (M9 agarose with glucose and arabinose). The parameter that varies between these 4 wells is the concentration of α-methyl glucose. Figure 6B shows the difference of *araBAD* expression between those wells (supplementary movie 3). In well 1, there is no α-methyl glucose: cells grow fast while there is glucose and when this latter is exhausted, they induce synchronously the expression of *araBAD*. In well 2, the α-methyl glucose has slightly reduced the glycolytic flux and some cells induce the *araBAD* expression before glucose exhaustion. In well 3, the glycolytic flux is strongly reduced, growth is slow and most cells induce the *araBAD* expression before glucose exhaustion. In the last well, the concentration of α-methyl glucose is so high that cells appear to be poisoned, but all induce *araBAD* expression before glucose exhaustion. This experiment demonstrates that we can control carbon catabolite repression by tuning the glycolytic flux. The heterogeneity of *araBAD* expression between the cells is a well-known phenomenon (Megerle *et al.*, 2008). This experiment also points out a way to tune the distribution of *araBAD* expression in the population of cells.

Discussion

In this study, we have explored the mechanism of carbon catabolite repression by studying the regulation of transcription of Crp-cAMP dependent operons in different genetic contexts and in different conditions. We monitored *in vivo*, in real time and with a high temporal resolution, the activity of several Crp-cAMP dependent promoters. Different strategies of controls have been implemented to assess the robustness of our results: we used two reporters systems (GFP/luciferase) and we performed the experiments both at the population level (liquid medium) and at the single cell level (solid medium).

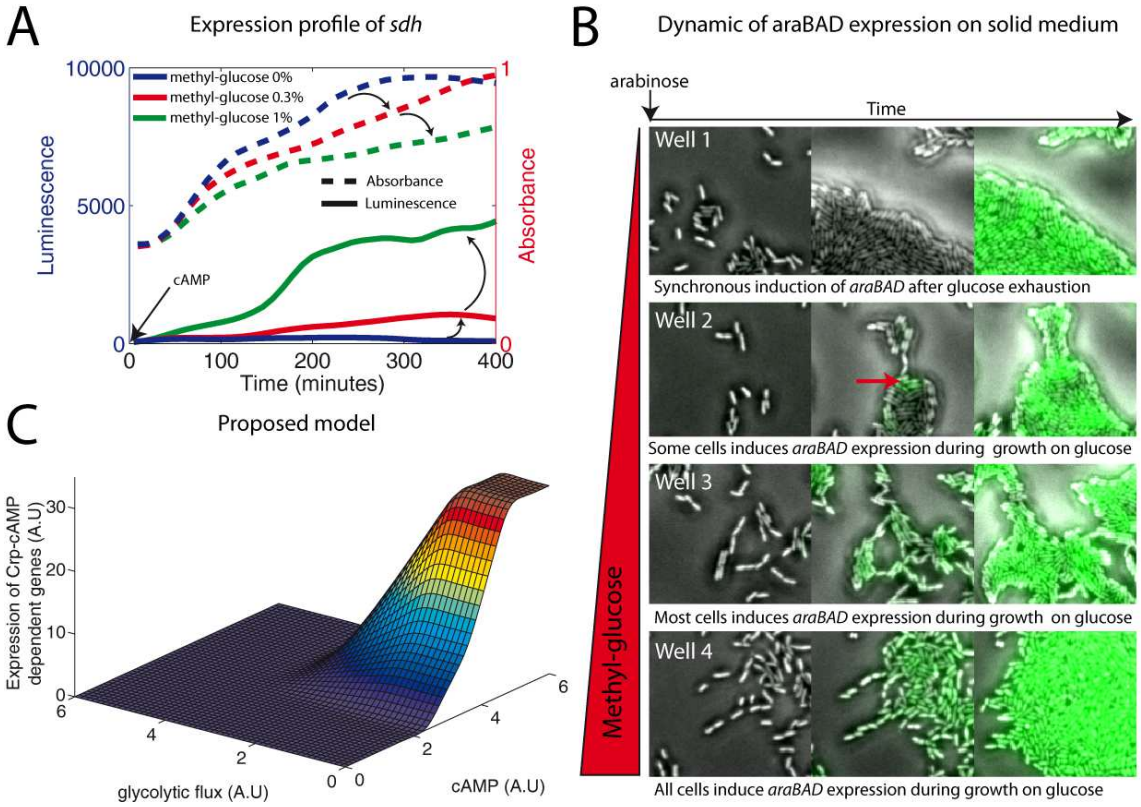

Figure 6. Role of the glycolytic flux in carbon catabolite repression. (A) Bacteria (*cyaA* strain) were grown on a minimal medium supplemented with 0.03% glucose, 0.03% acetate and 600µM cAMP. Non-metabolizable α-methyl-glucose controls the growth of cells by competing for glucose uptake and catabolism. The resulting reduction of the glycolytic flux induces the *sdh* promoter activity in a dose dependent manner. (B) *araBAD* expression of cells growing on a solid medium composed of glucose (0.06%) and arabinose (0.005%). 4 wells are filmed in parallel. Only the concentration of α-methyl-glucose varied between the wells (0%, 0.3%, 1% and 3%). This parameter tunes the level of *araBAD* expression, but also the proportion of cells that express *araBAD*. (C) A model of carbon catabolite repression: the combination of a sufficient concentration of cAMP and a sufficient reduction of the glycolytic flux is needed to synergistically induce CCR dependent genes.

Is there any role for cAMP in carbon catabolite repression of the *Escherichia coli* Crp-cAMP operons?

This question has been addressed in 2008 for the case of the *lac* operon (Gorke & Stulke, 2008b; Crasnier-Mednansky, 2008; Narang, 2009a). In 1998, Inada et al. have demonstrated than glucose repression is maintained both in a *cyaA* strain complemented with cAMP and in the double mutant *cyaA crp** (Inada *et al.*, 1996). The authors concluded that inducer exclusion, blocking the entry of lactose, was the main contributor to carbon catabolite repression when *E. coli* grows in the presence of both glucose and lactose. Later, Narang (Narang, 2009b), by an accurate and exhaustive review of four decades of results, has pointed out that the small difference of levels of cAMP during growth on glucose compared to growth on lactose cannot account for the large repression of β-galactosidase on glucose. These results clearly challenge the cAMP model.

In this context, our results demonstrate: (i) that cAMP is necessary in the wild-type strain to increase transcription of Crp-cAMP dependent promoters. Adding cAMP in the medium complements the *cyaA* mutant and thus increases the expression of all operons studied in this work ; (ii) that, in the wild-type strain, cAMP is not sufficient for explaining carbon catabolite repression: adding cAMP to the medium fails to retrieve full gene expression while glucose is still present ; (iii) that cAMP is not necessary in a *cyaA crr crp** strain. This strain, devoid of all supposedly essential components of CCR, still shows strong glucose repression.

Is there a role for the PTS in carbon catabolite repression of the *Escherichia coli* Crp-cAMP operons?

Dephosphorylation of enzyme IIA_{glc} is thought to be the main cause of glucose repression. However, non-PTS carbohydrates, such as glucose-6-phosphate, also induce a strong catabolite repression. Hogema et al. have shown that glucose-6-phosphate added in the medium induces the dephosphorylation of $EIIA_{glc}$. Yet, they have also observed that glucose-6-phosphate is still able to repress catabolic operons in a *crr* strain (Hogema *et al.*, 1997). This last result clearly challenges the main role of enzyme $EIIA_{glc}$ in carbon catabolite repression.

In this context, our results: (i) demonstrate that, in the wild-type strain, the PTS is necessary for increasing transcription of the *acs* promoter: the *ptsI* and *crr* strains show a very low level of *acs*

transcription, except when exogenous cAMP is added to the medium ; (ii) demonstrate that the PTS is involved in the control of cAMP production: the activation of adenylate cyclase by the $EIIA_{glc}$ enzyme remains poorly understood as it seems to require additional components not yet discovered (Park *et al.*, 2006); (iii) demonstrate that the phosphate transfer system (PTS) is not sufficient to account for carbon catabolite repression: a strong carbon catabolite repression still occurs in a *crr* strain complemented with exogenous cAMP ; (iv) demonstrate that the PTS is not necessary in a *crr cyaA crp** strain for carbon catabolite repression: this strain still shows glucose repression.

Mechanism of carbon catabolite repression

How can we reconcile these apparently contradictory results? The rise of the intracellular concentration of cAMP when glucose is exhausted is *correlated* with the induction of Crp-cAMP dependent operons. The simplest and most intuitive explanation is that this increase in cAMP concentration is *responsible* for the induction of Crp-cAMP dependent operons (Epstein *et al.*, 1975). However, there still remains the counter-intuitive possibility that those events, although correlated, may not be linked by a cause and effect relationship. Our experiments show that the sequence "glucose exhaustion - rise in cAMP concentration - transcription of Crp-cAMP operons" is not correct, or at least incomplete. The model that we propose in figure 6C assumes that the transcription of CCR-controlled Crp-cAMP operons requires two factors: a sufficient concentration of cAMP and a reduction of the glycolytic flux.

The glycolytic flux would have to be detected by an as yet unknown sensor, detecting the concentration of an as yet unidentified metabolite. Different CCR-controlled promoters differ by their sensitivity to this signal and their sensitivity to the concentration of cAMP. Only when both conditions are met will the corresponding promoter be fully induced. The *acs* promoter, for example, requires a high concentration of cAMP, whereas the *uhpT* promoter is already induced at a much lower concentration. Therefore, in the presence of glucose-6-phosphate, growth rate and glycolytic flux are high, and the cAMP concentration is low. With a qualitatively identical regulation mechanism (Figure 6C), but different parameters, the *acs* promoter is turned off in these conditions, whereas the *uhpT* promoter is fully active. When glucose-6-phosphate is exhausted, growth rate decreases and the concentration of cAMP increases. *Acs* is now fully expressed. According to the model, *uhpT* should still be maximally expressed. However, this promoter also requires the inducer glucose-6-phosphate for full activity, and this inducer is no longer present. *UhpT* transcription is thus shut off (see supplementary information: the metabolic toggle switch). Even though

the underlying regulatory mechanism is the same for both promoters, the observed expression pattern is very different. The same is true for the *gntT* promoter, responding to gluconate. This dual antagonistic effect of inducers has previously been described in a study that also clearly points out problems with the cAMP model (Peekhaus & Conway, 1998).

A unifying view of carbon catabolite repression

We therefore propose that carbon catabolite repression comprises the "classical" mechanisms involving the PTS and the concentration of cAMP. In addition, a yet unknown sensor detects the "metabolic state" of the cell, closely related to the glycolytic flux. The unknown mechanism (i) is independent of the concentration of cAMP, the phosphorylation state of $EIIA_{glc}$ and the concentration of Crp; (ii) is correlated with the glycolytic flux, but unrelated to PTS functioning, and thus unrelated to the PEP/pyruvate ratio (Hogema *et al.*, 1998); (iii) functions as a rapid switch, at the scale of signal transduction events. The activity of the CCR-controlled promoters results then from the synergistic activation of the target promoter by these two signals. We are currently working on the identification of this unknown signal and the way this signal is ultimately transmitted to the activator, Crp.

Materials and Methods

Strains

The mutant strains come from the Keio collection (Baba *et al.*, 2006). The wild-type strain (*E. coli* K-12 strain BW25113) has a well-defined pedigree and has not been subjected to mutagens (Datsenko & Wanner, 2000). This wild-type strain contains a deletion of the *araBAD* operon. Consequently, arabinose, that we add as inducer, can't be used as a carbon source and thus its concentration remains constant. We removed several kanamycin cassettes from mutants using flp recombinase to allow antibiotics compatibility with the plasmids derived from the Zaslaver collection. The *cyaA crr* double mutant was constructed by removing the kanamycin resistance cassette of the *cyaA* strain. Then we transduced (P1 phage transduction) the kanamycin resistance cassette of the *crr* strain in the *cyaA* strain and then remove again the new kanamycin cassette. The *crp** strain comes from a gift of Pr. Patrick Cirino (Khankal *et al.*, 2009). The *crp** gene was introduced into the *cyaA* mutant and the *cyaA crr* double mutant by P1 phage transduction using a tetracycline resistance cassette.

Plasmids

We have used home-made p-acs-lux, p-acs-gfp and p-sdh-lux plasmids whose construction has already been described. We have also used the p-uhpT-gfp, p-araBAD-gfp, p-sdh-gfp, p-gntT-gfp, p-glpA-gfp coming from the Zaslaver collection (Zaslaver *et al.*, 2006). Note that *luxCDABE* genes come from Xenorhabdus luminescens and the *gfp* variant used (*gfpmut2*) is highly stable and non toxic. The plasmid carries either a colE1 replication origin and an ampicillin resistance cassette (*lux*) or a pSC101 origin and a kanamycin resistance cassette (*gfp*). The two types of plasmid are fully compatible and they include a strong ribosome binding site just upstream the reporter gene.

The *crp* gene was amplified by PCR using primers 5'-ATAAGGTACCATGGTGCTTGGCAAAC CGC-3'(kpnI site underlined) and 5'-TCCGGGATCCTTAACGAGTGCCGTAAAC-3'(bamH1 site underlined) using BW25113 strain as template. The PCR product, digested with kpn1 and bamH1, was inserted into the previously described p-tet-lux plasmid to make p-tet-crp.

Measurement of gene expression at the population level

Dynamic measures of gene expression were performed by growing cells in a minimal medium (M9) with the indicated carbon sources during several hours within a microplate reader (fusion alpha FP-HT). This micro plate reader allows growth with shaking and temperature control (37°C). It measure cell density (OD at 600nm), luminescence and fluorescence (485nm/520nm) at a high temporal resolution (5 minutes). The microplate contains 180μl of medium and 20μl (dilution 10) of cells in stationary phase (pre-culture LB+corresponding antibiotics). The main parameters that differ between experiments are the cAMP, inducers, anhydrotetracycline (aTc), glucose and acetate concentrations (indicated in the legend of figures).

Measurement of gene expression at the single cell level

Briefly (details of each experiment are provided in the supplementary section), we add 1 ml of a minimal medium M9 agarose 0.8% (w/v) (with the indicated concentration of carbon sources, inducers, cAMP and antibiotics) into a 6 wells micro plate. After polymerization of agarose, the previous medium becomes solid and we add over the indicated volume of liquid M9 containing cells in stationary or exponential phase (pre-culture LB + antibiotics). We let some cells fall on the solid medium during 10 minutes

and then remove this liquid medium. Cells, which are placed on the top of the agar pad, get a perfect access to oxygen and begin to form micro-colonies. Image acquisition is performed using a 40X objective of the Olympus IX81 motorized inverted microscope. Note that the solid medium is placed between the objective (down) and the cells (up). Fluorescence and phase contrast images are collected each ten minutes during several hours at room temperature. Image processing is limited to the superposition of phase contrast and fluorescence images and to the normalization between minimum and maximum pixel intensity of the images. We have used home-made scripts and the image processing toolbox of Matlab.

Acknowledgments

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Supplementary information

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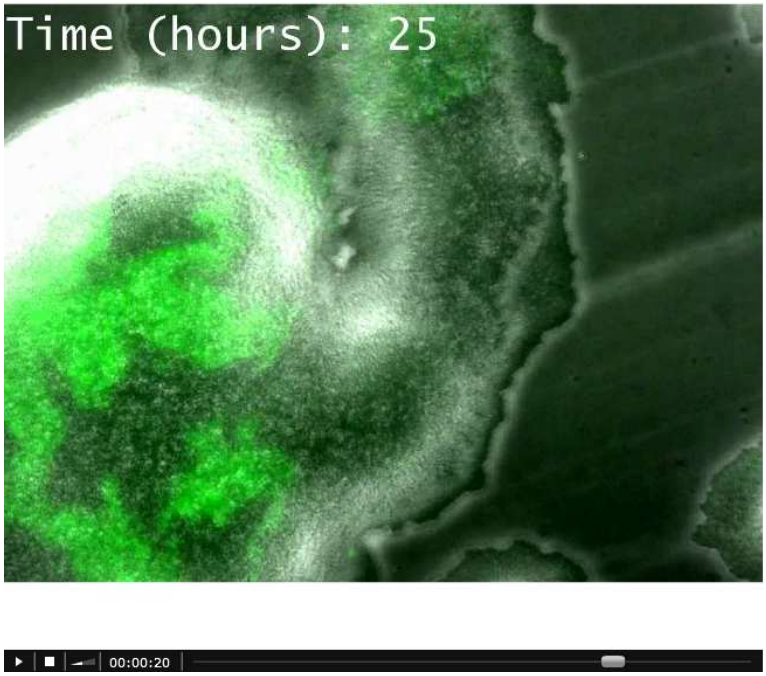
1 Video 1: Wt p-acs-gfp vs *cyaA*- p-acs-gfp



Materials and methods

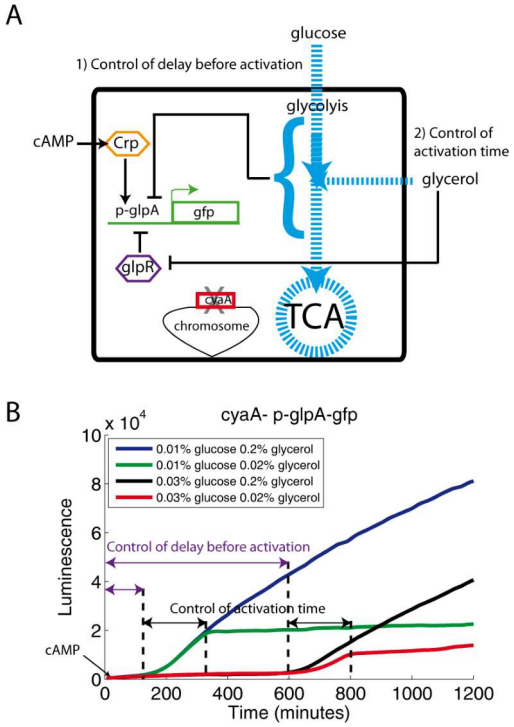
We added 1 ml of a minimal medium M9, containing 0.8% (w/v) agarose, 0.3% glucose, and 50μg/ml kanamycin into a well of a 6 wells-microplate. After polymerization of the agarose, we overlaid the now solid medium with 400μl of M9 medium and a mixture of two bacterial strains precultured in LB: 200μl of the wild-type strain containing the p-acs-gfp plasmid and 200μl of the *cyaA*- strain containing the p-acs-gfp plasmid. The precultured cells were still in exponential phase (OD>0.5) and their growth

medium, LB, was thus still "fresh". Part of the cells sedimented onto the solid medium during a 10 minute incubation. We then removed the liquid medium. Note that the remaining traces of fresh LB medium may "help" the growth. The remaining cells are thus located on the top of the agar pad; they get a perfect access to oxygen and begin to form microcolonies. Image acquisition was performed using 40X objective of the Olympus IX81 motorized inverted microscope. The solid medium is located between the objective (down) and the cells (up). Fluorescence and phase contrast images were collected every ten minutes for several hours at room temperature. Image processing was limited to the superposition of phase contrast and fluorescence images and to the normalization between minimum and maximum pixel intensity of the entire batch of images. All image treatments were done with home-made scripts and the image processing toolbox of Matlab.

Results

Towards the end of the video we observe a strong increase of fluorescence in parts of the microcolony, corresponding to the induction of *acs* transcription. The time of fluorescence increase coincides with the growth arrest of the microcolony, probably due to glucose exhaustion. Other parts of the microcolony, corresponding to the *cyaA*- strain, remain dark (no fluorescence increase), as expected. In this video, we also observed six cells that were fluorescent already at the beginning of the experiment. Our interpretation of this observation is that these cells have a strongly reduced glycolytic flux (probably due to an unfavorable local environment, spontaneaous mutations, the particular history of these cells, or a bet-hedging strategy of the cell), and consequently express *acs*.

2 The *glpA* promoter



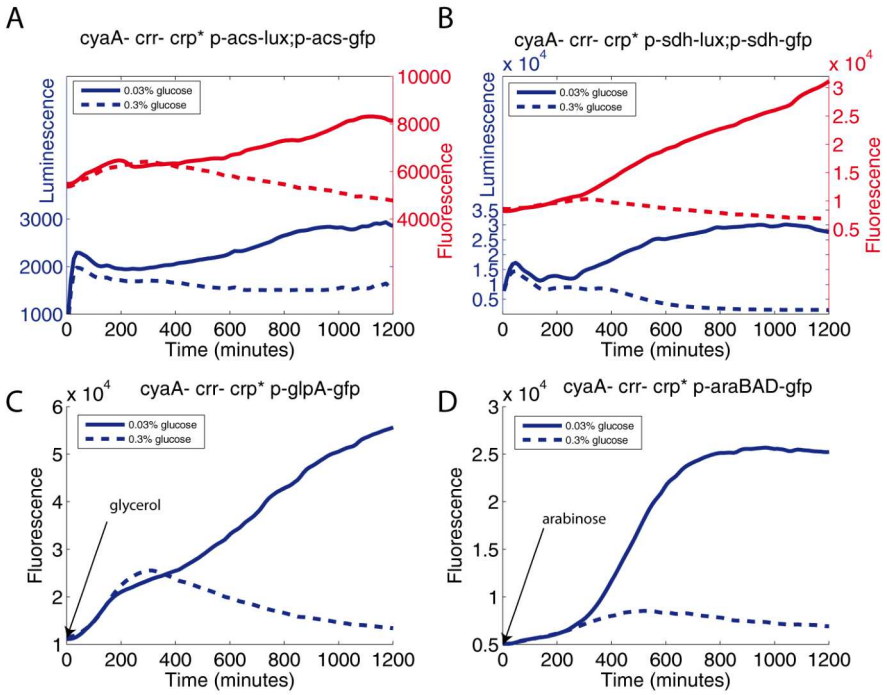
Materials and methods

A *cyaA*- strain transformed with the *p-glpA-gfp* plasmid (A) was grown in M9 medium containing glucose, glycerol and cAMP (500μM) (B). In this experiment, we have centrifuged the LB pre-culture and replaced the LB by an equivalent volume of fresh M9 before the 10-fold dilution of the cells.

Results

This promoter behaves as the *acs*, *sdh* and *araBAD* promoters. Glycerol and cAMP, added from the beginning of the kinetics, are not sufficient to induce the expression of *glpA* while glucose is still present. This behavior may be interesting for synthetic biology: glucose represses the activity of the *glpA* promoter and thus allows controlling the delay before the activation. Once glucose is exhausted, glycerol activates the promoter by inactivating the GlpR repressor: we observe gene expression. When glycerol is also exhausted, the activity of the *glpA* promoter drops again to zero.

3 Carbon catabolite repression in the *cyaA crr crp** strain



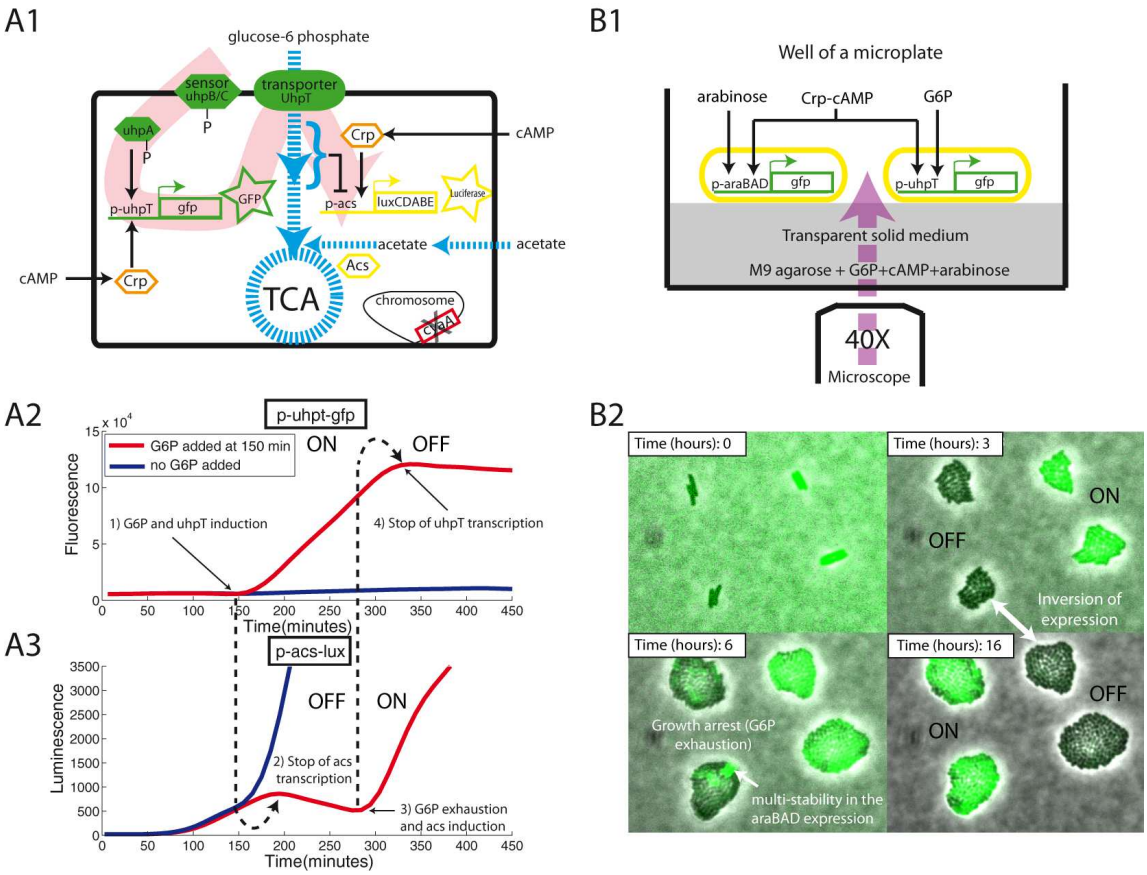
Materials and methods

The *cyaA crr crp** strain, transformed with the indicated plasmids (transcriptional fusions), were grown (10-fold dilution of the preculture) either in an LB medium supplemented with 0.03% glucose or in a LB medium supplemented with 0.3% glucose. Inducers were added when needed: Glycerol (0.08%) or arabinose (0.001%).

Results

In this experiment, we measure the activity of 4 Crp-cAMP dependent promoters (*acs*, *sdh*, *glpA*, and *araBAD*) in the "cAMP and EIIA$_{glc}$ free" strain (*cyaA crr crp**). We compare the level of fluorescence of theses strains growing in LB either with a low glucose concentration or a high glucose concentration. In all cases (A, B, C and D) we observe that the level of fluorescence (or luminescence) increases in the low glucose condition compared to the high glucose condition.

4 A synthetic toggle switch controlled by the glycolytic flux



Description of the figure

(A1) Schematic of a *cyaA* mutant transformed with two transcriptional fusions: p-acs-lux and p-uhpT-gfp. (A2 and A3) Bacteria were grown on a minimal medium supplemented with 0.03% glucose and 0.03% acetate. At 100 minutes, we observed the first *acs* induction. Adding G6P (0.03%) to the medium triggers *uhpT* expression, leading to a strong glycolytic flux that stops *acs* expression. Once exhausted, G6P cannot longer activate *uhpT* transcription and a new phase of *acs* induction begins. (B1) Schematic of a well containing a solid minimal medium containing the following carbon sources and complements: G6P (0.06%), cAMP (750μM), arabinose (0.01%), and acetate (0.03%). Two types of *cyaA* cells monitor either (i) the expression of *araBAD* or (ii) the expression of *uhpT*. (B2) Frames of the time lapse microscopy:

the two microcolonies on the left report *araBAD* expression whereas those on the right report *uhpT* expression. Note the inversion of expression after growth arrest (presence/absence of the glycolytic flux). Note also the heterogeneity of fluorescence among cells reporting the *araBAD* expression. This phenomenon of multi-stability is well-known : it is due to a positive feedback loop involving the arabinose transporter.

Results

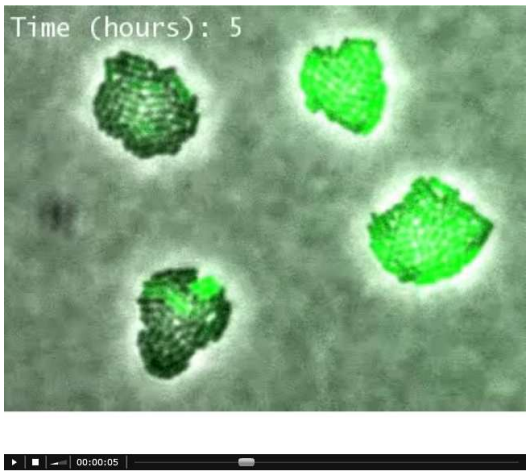
Our experiments suggest that CCR-controlled promoters are activated as a function of a metabolic parameter related to growth rate, e.g., the glycolytic flux. The different CCR-controlled promoters differ by their cofactor requirements (inducers such as arabinose or glucose-6-phosphate) and their sensitivity to cAMP. At the two extremes are the *acs* and *uhpT* promoters. To visualize the complementary behavior of these two promoters, we have constructed a synthetic toggle switch.

Both promoters are dependent on Crp-cAMP, but to different extents. Whereas *acs* requires a high concentration of cAMP for maximal activity, *uhpT* already reaches maximal activity at a much lower concentration of cAMP, but in the presence of the inducer, glucose-6-phosphate. The *acs* promoter is repressed by glucose-6-phosphate, an excellent carbon source allowing fast growth of *E. coli,* whereas this compound is the indispensable inducer of the *uhpT* promoter. In our system (figure A1), two transciptional fusions (p-acs-lux and p-uhpT-gfp) monitor the activity of their respective promoters in a *cyaA* strain. As before, this strain induces *acs* expresssion when glucose is exhausted (luminescence; figure A3). The *uhpT* promoter remains inactive because the inducer is absent. We then added glucose-6-phosphate during the induction phase of the *acs* promoter, resulting in the activation of the *uhpT* promoter (increase of fluorescence; figure A2). At the same time, glucose-6-phosphate now serves as an excellent carbon source, increasing the glycolytic flux and shutting down the *acs* promoter. Once glucose-6-phosphate is exhausted, *acs* transcription is again induced and *uhpT* transcription is stopped. The control experiment, without addition of glucose-6-phosphate, shows no transcription of *uhpT* and no arrest of *acs* transcription, as expected.

A similar switch can be observed at the single-cell level (figure B1; supplementary movie 2). We filmed two types of *cyaA* cells growing on the same agar patch, using glucose-6-phosphate as a carbon source: the first cell type reported the *araBAD* expression (p-araBAD-gfp) that occurs after glucose-6-phosphate exhaustion, whereas the second cell type reported *uhpT* expression (p-uhpT-gfp) that is induced in

the presence of glucose-6-phosphate. All inducers are present from the beginning of the experiment (cAMP, glucose-6-phosphate and arabinose). In figure B2, we show the inversion of expression of GFP as glucose-6-phosphate is being consumed. Cells reporting *uhpT* expression (on the right) are induced during the growth phase, whereas cells reporting *araBAD* expression are induced after exhaustion of glucose-6-phosphate.

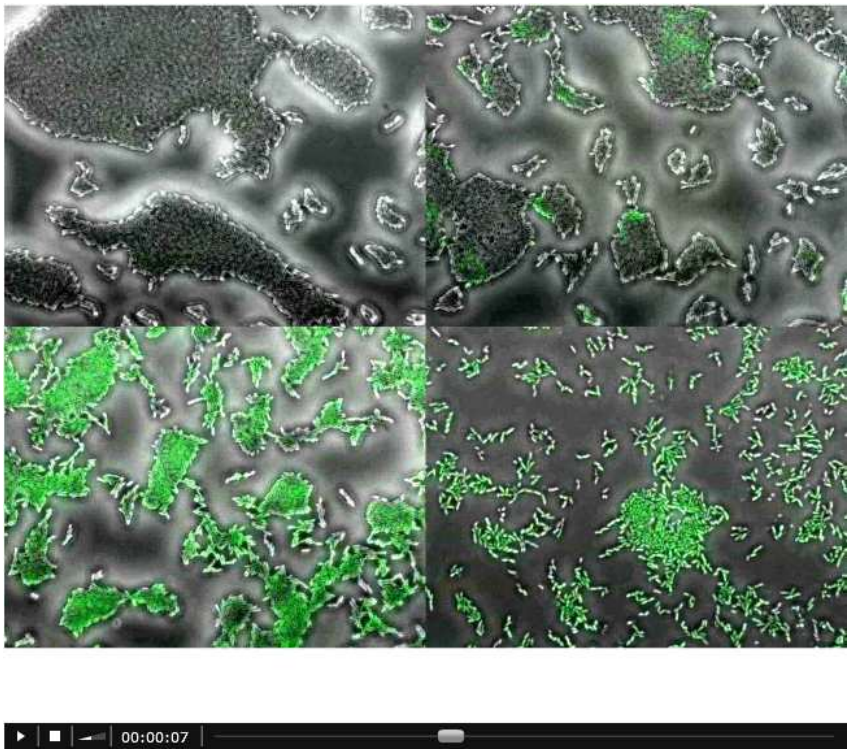
Video 2: *cyaA*- p-araBAD-gfp vs *cyaA*- p-uhpT-gfp



Materials and methods

This experiment used the same protocol as that used for Video 1, with the following experimental conditions. The medium used was M9 agarose 0.8%, sodium acetate 0.03%, glucose-6-phosphate 0.06%, cAMP 750μM, arabinose 0.01%, and kanamycin 50μg/ml. We used *cyaA*- strains either with the p-araBAD-gfp plasmid or the p-uhpT-gfp plasmid. After polymerization of the agarose, we add over 1 ml of M9 and 20μl (dilution 50-fold) of each strain, pre-cultured in LB (stationary phase). After having removed the liquid medium, the micro-plate was placed at 37°C for 30 minutes before beginning of the image acquisition (this step facilitates automatic detection of the focus). The fluorescence intensity was not normalized between minimum and maximum pixel intensity of the entire batch of images, but for each image independently. This allows a simpler comparison of the level of fluorescence of the two strains. However, the resulting pictures suggest that GFP is degraded during the experiment, which is not the case.

5 Video 3: glycolytic flux and catabolite repression



Materials and methods

We used the same method as the one of Video 1 with the following modifications. The medium used is M9 agarose 0.8%, glucose 0.06%, and arabinose 0.005%. We used a *sucB* mutant transformed with the *p-araBAD-gfp* plasmid. This strain is used because it can not grow on the secreted acetate. This leads to a very sudden growth arrest when glucose is exhausted (see the video). In this experiment, we filmed four wells in parallel. The only difference between the wells is the concentration of α-methyl-glucose (top left: 0%; top right: 0.3%; bottom left: 1%; bottom right: 3%). We assembled the four videos with Simulink (Video and Image Processing Blockset).

Results

On the top left of the video (Well 1), there is no α-methyl glucose: cells grew fast in the presence of glucose. After glucose exhaustion, they activate synchronously the *araBAD* promoter (rise of fluorescence). On

the top right of the video (Well 2), the α-methyl glucose has slightly reduced the glycolytic flux and some cells induce the *araBAD* expression before glucose exhaustion. On the bottom left of the video (Well 3), the glycolytic flux is strongly reduced, the growth is slow and most cells induce *araBAD* expression before glucose exhaustion. In the last well (bottom right), the α-methyl glucose concentration is so high that cells seem poisoned (very little growth), but all induce *araBAD* expression before the glucose exhaustion.

6 Critical parameters to reproduce our results

Given the contradictory results found in the literature about glucose repression, we provide some additional guidelines. Our results are most clearcut for the following experimental conditions:

- using a *cyaA*- strain with exogenous cAMP (or a *crp** strain; and in this case, do not add cAMP).
- using either minimal medium or LB (begin with minimal medium, and switch to LB in case of problem). In our experiments, the transition from pre-culture (LB) to culture is done with a low dilution factor (factor of 10: 180μl of medium + 20μl of pre-culture). This ensures a rapid exhaustion of the carbon sources.
- performing the kinetics in micro-plate in order to easily measure many different concentrations of:
  - cAMP
  - inducers (if needed)
  - Glucose: best results are obtained by comparing 0.03% glucose with 0.3% glucose. These conditions focus on what happen when glucose is exhausted (first concentration), compared to continued growth on glucose (second concentration).
- adding an alternative carbon source to ensure correct transcription/translation capabilities when glucose is exhausted. This carbon source must permit very slow growth (best results with sodium acetate 0.03% w/v).

Chapitre IV

A synthetic *Escherichia coli* communication system mediated by extracellular cyclic AMP

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Abstract

In *Escherichia coli*, intracellular cAMP is involved in carbon catabolite repression. It binds to the cAMP receptor protein (CRP) to control the transcriptional of more than 220 operons. *Escherichia coli* also actively exports cAMP into the growth medium where this molecule reaches non-negligible concentrations. However its role is not understood. In this study, we have engineered strains capable of communicating with each other by way of extracellular cAMP. We propose this new communication module to the synthetic biology community and we discuss its potential physiologic role in natural environment.

Introduction

3'-5'-cyclic adenosine monophosphate (cAMP) is a second messenger performing intracellular signaling in many organisms. Discovered in the 60s, the importance attributed to this compound has never stopped growing (Makman & Sutherland, 1965). The number of publications involving cAMP now exceeds 100000. In humans, the intracellular cAMP-dependent pathway is involved in the regulation of many biological processes ranging from glycogen metabolism to synaptic transmission and the control of heart rate. The deregulation of concentrations of intracellular cAMP is observed in many diseases such as cholera, anthrax, cancers or neurological disorders.

In addition to its role as an intracellular second messenger, cAMP also acts as first messenger (Konijn *et al.*, 1967). This function of cAMP was first discovered in the amoebae *Dictyostelium discoideum*, where extracellular cAMP mediates communication between cells by acting as a chemo-attractant. Periodic waves of extracellular cAMP allow these amoebae to convert from solitary predators into gregarious

community members (Tomchik & Devreotes, 1981). In the 1970s, some reports mentioned that cAMP was actively exported in the blood of humans (and then urine) where it could attain significant concentrations (in the range 10-100 nM) (Broadus *et al.*, 1970). Thirty years later, a wide body of experimental observations suggests that extracellular cAMP exerts endocrine and paracrine actions in several tissues (Bankir *et al.*, 2002).

In *Escherichia coli*, cAMP is involved in carbon catabolite repression (Gorke & Stulke, 2008; Narang, 2009) and binds to the cAMP receptor protein (CRP). The corresponding complex is a transcriptional factor controlling the expression of more than 220 operons (Keseler *et al.*, 2009). It has been known for a long time that *E. coli* actively exports cAMP into the growth medium (Goldenbaum & Hall, 1979; Makman & Sutherland, 1965). However, the role of this extracellular cAMP is not understood. In this study, we have engineered strains capable of communicating with each other by way of extracellular cAMP. Even though we have engineered the strains to clearly show communication via cAMP, the same mechanism could operate in a natural setting and provide a mean for communication between species that are phylogenetically only very distantly related.

Results

Communication on solid medium

To build an inter-cellular communication system, we engineered a sender strain capable of producing more than the usual amount of cAMP and a receiver strain able to sense this exported cAMP and to report its presence by a convenient signal. The simplest sender strain is the *crp* mutant, known to produce high extracellular cAMP level compared to a wild-type strain (Potter *et al.*, 1974). The receiver strain harbors a deletion of the *cyaA* gene (encoding the cAMP biosynthetic enzyme adenylate cyclase) in order to prevent any cAMP production. This *cyaA* mutant also contains a "bio-sensor" that converts the cAMP concentration into a luminescence signal. Placed on a plasmid, this biosensor is a transcriptional fusion, i.e, a promoter fused to a reporter gene. We chose the Crp-cAMP dependent *sdhCDAB* promoter (Nam *et al.*, 2005) and fused it upstream of the *luxCDABE* operon coding for bacterial luciferase (Van Dyk *et al.*, 2001).

To demonstrate inter-cellular communication, we used an interface assay. We embedded the sender cells into agarose gel containing minimal glucose medium on a Petri dish. The solid agarose gel containing

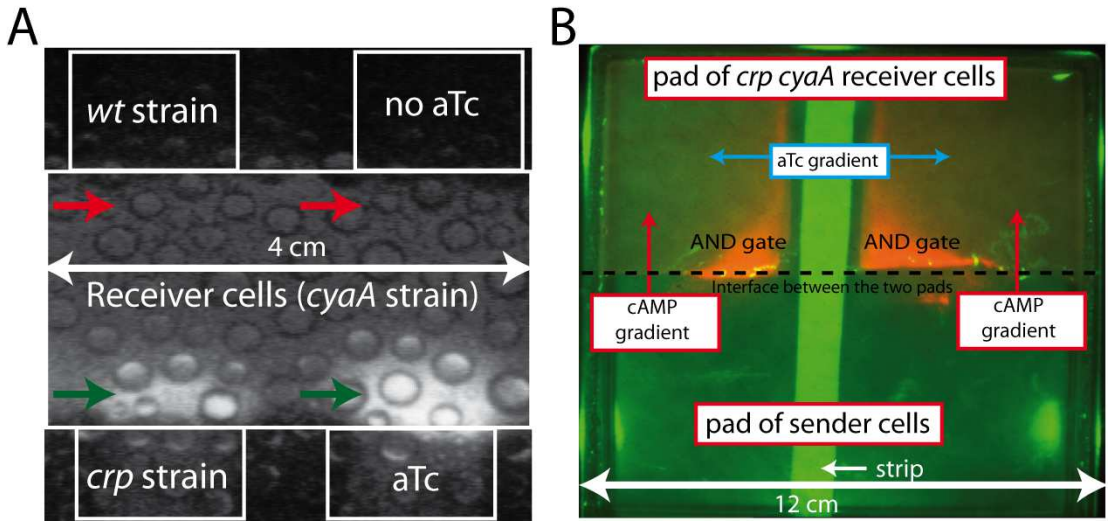

Figure 1. Demonstration of communication in solid medium. (A) Luminescence picture (seen from above) of a solid medium in a Petri dish acquired in a dark chamber with a sensitive camera (the round droplets are due to condensation on the lid). In the middle, a long rectangular pad of *cyaA* receiver cells containing the luminescence biosensor produces a basal level of light. On the top left, the interface between the receiver pad and the wild-type pad does not produce any rise of light (red arrow). Conversely, on the bottom left, a halo of light is observed at the interface between the "*crp* cells" pad and the "receiver cells" pad (green arrow). On the bottom right, sender cells overexpress adenylate cyclase (by the way of aTc) and a halo of light is observed at the interface (green arrow). On the top right, no halo appears because adenylate cyclase is not expressed (absence of aTc) (red arrow). (B) Picture of a Petri dish seen from above. Luminescence data (red false color) and photo of a Petri dish (green false color) are overlaid. One half of the Petri dish contains an agar pad of sender cells producing cAMP which diffuses in the agar pad of receiver cells (second half). At the middle, and perpendicular to the interface, a thin strip of cellulose soaked with anhydrotetracycline (aTc) is deposited on the agar and allows expression of Crp. Two bright areas (on either side of the strip) appear where the presence of both optimal aTc and cAMP levels allow formation of the Crp-cAMP complex in the receiver cells, and thus luciferase transcription.

the sender bacteria is brought into contact with a similar pad containing receiver cells. The Petri dish is placed in a heated, dark chamber and filmed for 40 hours using a sensitive CCD camera (see supplementary movie 1). An intense "halo" of luminescence appears in the area of contact between the "receiver cells" and the *crp* strain, called "sender cells" (Figure 1A; bottom left side). cAMP produced by the "sender area" has diffused into the "receiver area" and elicited the expected response: entering the receiver cells, binding to Crp and activating the *sdh* promoter, thereby inducing the expression of luciferase. By contrast, no "halo" appears at the interface between receiver cells and a wild-type strain (Figure 1A; top left side). This observation shows that a wild-type strain does not produce a sufficient amount of extracellular cAMP to allow the detection of communication.

Communication could be due to the exchange of any molecule, not necessarily cAMP. In order to demonstrate that cAMP is the signaling molecule, we remove the gene coding for adenylate cyclase (*cyaA*) in the sender strain. We put into the *cyaA crp* double mutant a plasmid that expresses *cyaA* from an anhydrotectracycline (aTc) inducible promoter. This strain produces cAMP only when the inducer aTc is present in the medium. As expected, a halo of luminescence appears only at the interface of the pad containing aTc (Figure 1A; right bottom side versus right top side). This experiment demonstrates that the expression of adenylate cyclase in the sender strain is necessary for inducing transcription of luciferase in the receiver strain.

A second way to demonstrate that cAMP acts as an intercellular signaling molecule is to show that the presence of the *crp* gene in the receiver strain is needed for the appearance of the luminescence halo. We transformed a *cyaA crp* double mutant with the plasmid PR100, carrying a construct that over-expresses *cyaA*. This latter is under the control of the strong lambda pL promoter and the gene carries the canonical AUG initiation codon, instead of the less efficient TTG found in the chromosomal *cyaA* gene. With this expression system, adenylate cyclase constitutes approximately 30% of the cellular proteins. As a receiver strain we also use the *cyaA crp* double mutant containing two plasmids. The first one carries the luminescence biosensor and the second plasmid allows to tune the expression of Crp by the aTc inducible system. We juxtaposed two agar pads containing these two strains and we added, overlaid in the middle of the Petri dish, and perpendicular to the generated interface, a thin strip of paper soaked with a solution of aTc. Figure 1B shows that in the area where the aTc gradient (expression of Crp) and the cAMP gradient generated by the sender cells overlap, we observe a bright luminescence of the reporter plasmid. Activation of the reporter gene corresponds to the AND logic gate behavior of the

Crp-cAMP complex. Communication passes through the first messenger cAMP produced by sender cells and requires the Crp protein as a second component in the receiver cells.

Tuning the communication in liquid medium

We have shown that communication via cAMP functions on solid medium. Is the amount of excreted cAMP sufficient to produce communication in liquid medium? In order to obtain quantitative information, we decided to duplicate the reporter system. In addition to the luciferase reporter, we added to the receiver strain an equivalent gfp reporter, also driven by the *sdh* promoter (Zaslaver *et al.*, 2006), but carried on a plasmid with a different origin of replication (pSC101). We measure the *sdh* promoter activity independently with each reporter system in the same cell (Figure 2A1). This double reporter system eliminates all potential artifacts due to the reporter system or the plasmid.

We first tuned the strength of communication by controlling the expression of adenylate cyclase in the sender (Figure 2A1; see also the tridimensional simulation in supplementary movie 2). We grow sender cells (allowing the control of *cyaA* expression by aTc) and receiver cells in a minimal glucose medium for 24 hours. The culture grows in a microplate and we continuously monitor luminescence, fluorescence and optical density. At specific points of the kinetics, we collected samples to measure extracellular cAMP by an immunoassay kit. Figure 2A2 shows that the extracellular cAMP concentration is proportional to the aTc level controlling adenylate cyclase expression in the sender cells. The increase of extracellular cAMP over time is gradual and reaches 80 μM after about 20h of growth.

Figures 2A3 and 2A4 show the corresponding induction of the *sdh* promoter after glucose exhaustion (at about 550 min). Both reporter systems, fluorescence and luminescence, show the same signal. This experiment shows that we can control the transcription of luciferase and GFP in receiver cells by tuning the adenylate cyclase level in the sender cells. Last, contrary to the gradual increase of extracellular cAMP, the strong increase of fluorescence and luminescence occurs only after glucose exhaustion (growth arrest indicated by an arrow on the figure 2A3). This result does not fit well with the classic model of carbon catabolite repression. Indeed, adenylate cyclase in sender cells should produce cAMP only after glucose exhaustion because its activator (the phosphorylated form of $EIIA_{glc}$.) is supposed to be present only in absence of glucose (Park *et al.*, 2006). Yet, the raise of extracellular cAMP is not linked to the glucose exhaustion and cAMP is already present at a high concentration in the medium well before *sdh* activation at 600 minutes (Figure 2A2 versus Figures 2A3 and 2A4). This result means that the

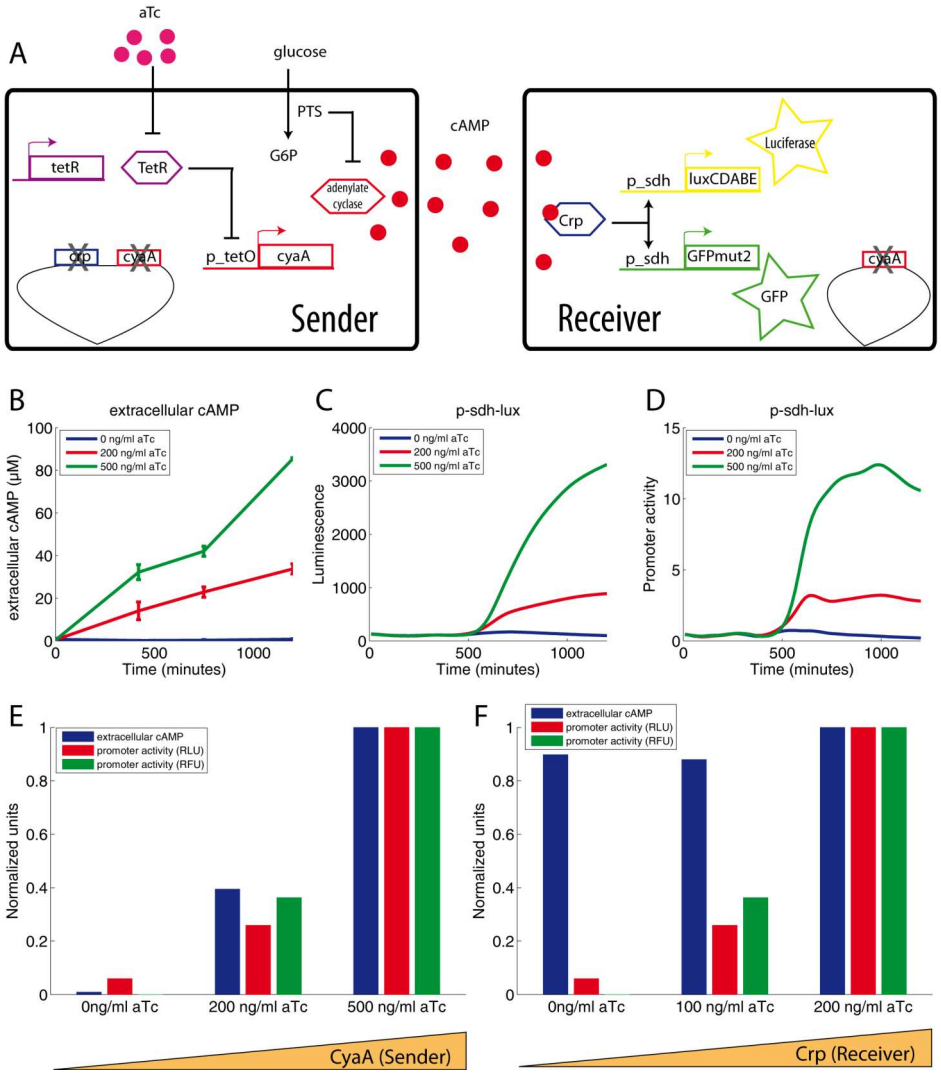

Figure 2. Tuning the communication in liquid medium. (A) Tuning the communication by controlling adenylate cyclase production in sender cells. (B) Extracellular cAMP is correlated with aTc levels. (C and D) The luminescence and fluorescence in the receiver cells after glucose exhaustion, when the transcription of *sdh* is induced, correlates with the aTc levels, and therefore the expression of adenylate cyclase in the sender cells and the concentration of cAMP in the medium. (E) Summary of the correlation between the concentration of cAMP in the medium and the response of the receiver cells at different concentrations of aTc; (F) Response of receiver cells with Crp expression under the control of the aTc-inducible promoter and in the presence of sender cells that produce constitutively high levels of cAMP (concentration measured in the medium: 150μM). Fluorescence is similar to the luminescence profile and is correlated to aTc levels after glucose exhaustion.

repression of *sdh* promoter activity by glucose is not due to the inactivation of adenylate cyclase in sender cells but rather due to an unknown mechanism that occurs in receiver cells.

Another way to tune the strength of communication consists in controlling the Crp concentration in the receiver cells (figure 2F). In this experiment, we used sender cells carrying the PR100 plasmid, which produces large amounts of cAMP. This microbial factory generates an extracellular cAMP concentration close to 150μM (data not shown). The fluorescence and luminescence levels follow the Crp concentration in the receiver cells (range of aTc) (Figure 2F) but only after glucose exhaustion. Once again, *sdh* promoter activity is repressed by a strong glucose repression. This latter is neither related to variation of extracellular cAMP level nor related to variation of Crp level. Another explanation must be sought.

Recovery of motility through communication

How could communication be used to elicit a physiological response? The *cyaA* mutant has many characteristic phenotypes. A *cyaA* mutant cannot utilize carbon sources such as maltose, lactose or acetate and it cannot produce flagella, resulting in a non-motile bacterium. This lack of motility is due to the absence of FlhDC, the principal regulator of bacterial flagellum biogenesis. The transcription of the *flhDC* operon is positively regulated by the cAMP-CRP complex (Soutourina *et al.*, 1999). If communication works, cAMP produced and exported by sender cells should complement the non-motile phenotype of *cyaA* receiver cells. We designed an assay based on the faculty of *Escherichia coli* to swim though a semi-solid medium (low concentration of agarose). We filled 3/4 of a well (6-wells microplate) with this semi-solid gel. The bottom left quarter also contains sender cells. Two drops of receiver cells where spotted onto this agarose gel: one at the center of the well close to sender cells, a second spot at the top left, far from sender cells (Figure 3A). We filmed the microplate for 50 hours (supplementary movie 3) and observed that the "center" spot spreads to the bottom left quarter containing sender cells as shown by luminescence (Figure 3B). By contrast, the "top left" spot does not move. We conclude that cAMP produced by sender cells complements the *cyaA* strain and allows the recovery of motility probably by allowing *flhDC* expression followed by flagella biosynthesis. Too far from cAMP, cells of the second spot do not recover motility. It has not escaped our attention that this system can be viewed as a synthetic guidance system.

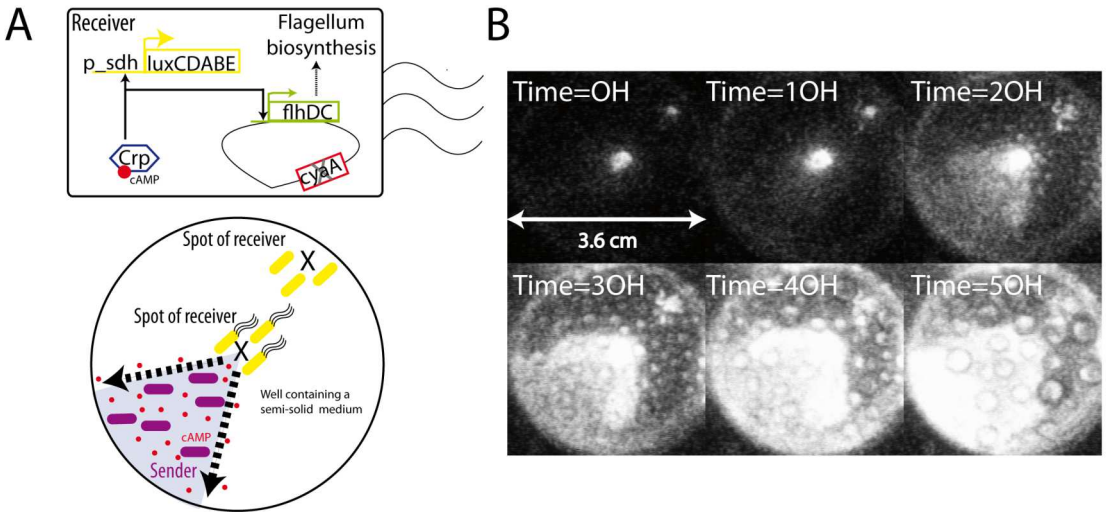

Figure 3. Recovery of motility allowed by the communication. (A) Schematics of a receiver cell and the geometry of the motility assay. The Crp-cAMP complex allows both luciferase expression and *flhDC* expression. The presence of cAMP produced by sender cells allows *cyaA* cells to produce flagella and to swim though the semi-solid medium. (B) Luminescence picture of the well showing the evolution of the luminescence pattern during two days (Round droplets are due to condensation on the lid). The spot of receiver cells in the middle of the well spreads to the left bottom quarter of the well because cAMP is present. In contrast, the "control" spot at the top right is too far from cAMP and cannot recover motility.

Discussion

A synthetic system

In this study, we have developed an easily tunable synthetic system allowing inter-cellular communication of *Escherichia coli* cells by the way of extracellular cAMP. We propose this system to the synthetic biology community and discuss these advantages and disadvantages. Contrary to other intercellular communication systems such as LuxI/LuxR from Vibrio fischeri or LasI/LasR from Pseudomonas aeruginosa (Balagadde *et al.*, 2008), the main default of our system is its absence of orthogonality. Indeed, it is not independent from the regulatory network of *E. coli*. On the contrary, the cAMP-CRP complex is considered as a "hub". The lack of orthogonality places our system at the edge between synthetic and system biology. Because our system is not uncoupled from natural regulatory network, it can be used as a tool to understand, disturb and control it. The huge knowledge on the cAMP-CRP complex can be used and embezzle to develop new synthetic functions: The high number of promoters controlled by Crp-cAMP offers a wide choice of different affinity binding site (Keseler *et al.*, 2009). The "hub" Crp-cAMP is already connected to a lot of different cellular modules (motility, chemotaxis, virulence, biofilm, stress resistance, carbon utilization) and composes a wide number of known network motifs such as coherent and incoherent feed forward loops (Alon, 2006). The connection of the Crp-cAMP "hub" to metabolism, via the mechanism of carbon catabolite repression, is a case study for system biology and different already existing models could be re-used (Bettenbrock *et al.*, 2006; Hardiman *et al.*, 2007; Kotte *et al.*, 2010; Nishio *et al.*, 2008). Last, cAMP is strongly ubiquitous: it's a messenger shared by most of the living species. Extracellular cAMP is thus a good candidate to perform synthetic inter-kingdom communication systems.

A microbial factory and a biosensor as new tools

In this study, we engineered a strain able to produce up to 150μM extracellular cAMP i.e well above the concentration reached by a wild-type strain (100nM-10μM) (Matin & Matin, 1982). It's still far from the concentration (30mM) produced by Microbacterium sp. no. 205 (Ishiyama, 1990). It is therefore unlikely that our strain is competitive on an industrial scale. However the current yield can probably be optimized by adapting the growth conditions or by strain improvement, for instance, by removing the phosphodiesterases (*cpda*) which has a reported Km of 500μM (Imamura *et al.*, 1996) or by modifying the

phosphate transfer system to improve adenylate cyclase activation (Gosset, 2005). In this study, we also described a "biosensor", a *cyaA* strain that convert exogenous cAMP concentration into luminescence and fluorescence signals. The lower limit of detectable concentrations is closed to 10μM. The active export of cAMP is probably the main reason explaining why the biosensor fails to easily report extracellular cAMP concentration yet equivalent to physiologic intracellular concentration in a wild-type strain. Removing this mechanism of export may increase the sensitivity of our biosensor to reach the Crp km. Last, the unknown phenomenon of glucose repression observed in the figure 2 but unrelated to cAMP concentration is a parameter that influences the output signals generated by our reporters. Understanding and removing this phenomenon may also increase the performance of the biosensor.

A potential physiologic system

In this study, we do not demonstrate that communication by cAMP is a physiologic system in *Escherichia coli*. Indeed wild-type cells produce a maximum extracellular cAMP level (5-10μM) which roughly corresponds to the minimum threshold of detection of our biosensor. Is that mean that the reachable extracellular cAMP concentration must not change drastically the gene expression of the population? But then why is cAMP exported in the medium? It has already be demonstrated that the cost of the cAMP export in term of ATP molecules is too high to only serve as a mechanism used to regulate intracellular cAMP level (Matin & Matin, 1982). Could we imagine that a tiny fraction of the population begins to sense the extracellular cAMP and expresses Crp-cAMP dependant operon as a bet hedging strategy? is *E. coli* uses its secreted extracellular cAMP to communicate with other species or less "anthropocentrically" to change their phenotypes? One track to search for physiologic communication process may be to look towards the natural environment of *E. coli*: the intestinal ecosystem. By way of conclusion, we invite the reader to watch the movie 4 (and the explanations in supplementary information) where we made an attempt in this direction.

Materials and Methods

Strains and plasmids

We used the *E. coli* K-12 strain BW25113 as "wild-type strain". This strain has a well-defined pedigree and has not been subjected to mutagens (Datsenko & Wanner, 2000). We used the *cyaA*- strain and the

crp- strain of the Keio collection (Baba *et al.*, 2006). We removed the kanamycin resistance cassette of the *cyaA*- strain using flp recombinase in order to allow utilization of the p-sdh-gfp plasmid which also harbors a kanamycin resistance cassette (Zaslaver *et al.*, 2006). The *cyaA*- *crp*- double mutant was constructed by P1 transduction of the spectinomycin resistance cassette of another *crp*- mutant (Soutourina *et al.*, 1999).

Precultures were always performed in LB medium with the appropriate antibiotics (ampicillin 100 μg/ml; kanamycin 50 μg/ml; chloramphenicol 30 μg/ml; spectinomycin 10 μg/ml) at 37°C under shaking with the exception of the strain harboring the PR100 plasmid (with the thermosensitive lambda promoter) which has grown at 30°C to prevent too strong expression of *cyaA* resulting in insoluble adenylate cyclase (Reddy *et al.*, 1989).

The gltA-sdhC intergenic region was amplified by PCR using primers 5'-TATC**CTCGAG**TTAAGGT CTCCTTAGCGCCTT-3' (xho1 site in bold) and 5'- TTCT**GAATTC**GAATAACGCCCACATGCTG TTC-3' (EcoRI in bold) using BW25113 strain as template and inserted into *luxCDABE* plasmid backbone without promoter to make p-sdh-lux.

The *crp* gene was amplified by PCR using primers 5'-ATAA**GGTACC**ATGGTGCTTGGCAAACC GC-3'(kpnI site in bold) and 5'-TCCG**GGATCC**TTAACGAGTGCCGTAAAC-3'(bamH1 site in bold) using BW25113 strain as template. The PCR product, digested with kpn1 and bamH1, was inserted into the previously described p-tet-lux plasmid to make p-tet-*crp*.

The *cyaA* gene was amplified by PCR using primers The 5'-GCGA**GGTACC**TTGTACCTCTATATT GAGACT-3' (kpnI site in bold) and 5'-ACAGTC**GCTAGC**TTAACTAGCATCAGCATAATTTTCACT GTAGTTTTCGTCGTTTGCTGCCGAAAAATATTGCTGTAATAGC-3' (nheI site in bold) using BW 25113 strain as template. The PCR product, digested with kpn1 and nheI, was inserted into the previously described p-tet-lux plasmid to make p-tet-*cyaA*. Note that the oligo introduces a degradation tag das+4 in 3' (AANDENYSENYADAS). This tag doesn't play any role in this study but it may significantly reduce the half-life of the corresponding adenylate cyclase (McGinness *et al.*, 2006). The PR100 plasmid was a gift from Pr. Reddy.

Solid media assay

We used a minimal medium with 0.3% (w/v) glucose and 0.8% (w/v) low melt agarose. After a washing step to remove antibiotics, we added, in the unsolidified medium and just before polymerization, the

receiver cells (dilution factor 1/10) or the sender cells (dilution factor 1/4) coming from LB pre-cultures. Interfaces were created step by step by pouring the unsolidified medium mix in the Petri dish (then sometimes, cutting to adjust final shape) and waiting for solidification. Then the next unsolidified medium mix was added so as to touch the previous pad to create the interface. After polymerization of the entire pad, the Petri dish was placed in a heated chamber and filmed during the appropriate time (days) by a sensitive CCD camera (photonic science). The thin strip of paper (whatman) in Figure 2 is saturated with 100 μl of a solution of anhydrotetracycline (1mg/ml).

Liquid media assay

Dynamic analyses were performed by growing cells during 24 hours in a 96-well microplate. The microplate reader (fusion alpha FP-HT) allows growth with shaking and temperature control (37°C). It measure cell density (OD at 600nm), luminescence or fluorescence (480nm/520nm) every 15 minutes. Each well contains 180 μl of minimal medium (M9) with 0.3% glucose (w/v) and with a range of anhydrotetracycline (0, 100, 200, 500 ng/ml). We added 10 μl of appropriate receiver cells and 40 μl of appropriate sender cells after a washing step to remove the antibiotics of the preculture (LB). Contrary to luminescence data which don't produce background, we subtracted a background from fluorescence data: this background is the fluorescence generated by the control (0 ng/ml aTc). Sample for extracellular cAMP measurement were collected at 0, 420, 750 and 1200 minutes. We used the chemiluminescent cAMP HTS immunoassay (Millipore). All measures were performed in triplicate except one outlier removed (at time 1200, 500 ng/ml, Figure 2).

Semi-solid media assay

The minimal medium (M9) contains 0.3% galactose and 0.3% low melt agarose. We added 4ml in a well of a 6 wells-micro plates. Once "semi-solidified", we were still able to sculpt the agar and we removed, by using a pipetman, one quarter of the semi-solid medium (0.8 ml) that we replaced by 0.8ml of the same medium but containing the sender cells (*crp-cyaA*- double mutant with the PR100 plasmid) (a pullet resuspended coming from the centrifugation of 2ml of preculture). Then we added, on the top of the agar, two spots (center and top left) of 1μl of receiver cells (concentrated 200X).

Image processing

All movies and images were processed using Matlab software. No specific image enhancement has been performed.

Acknowledgments

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Supplementary information

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1 Calibration of the p-sdh-lux biosensor

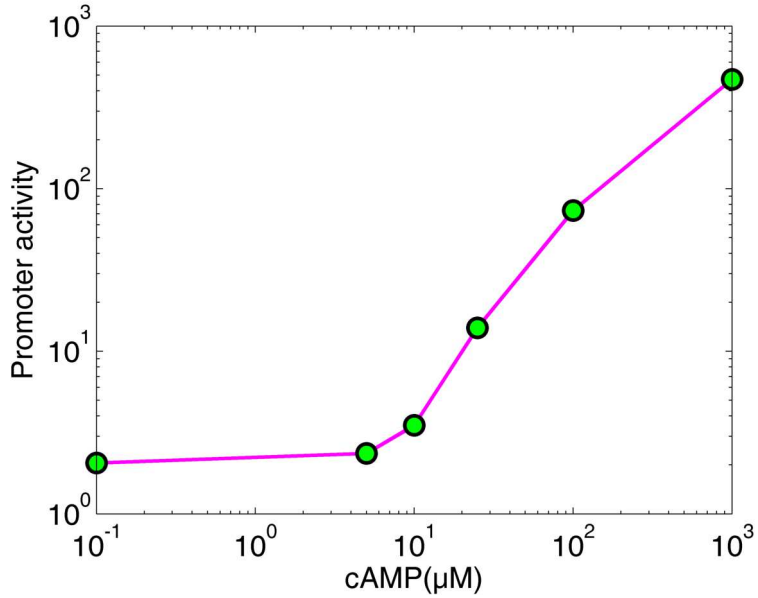

Figure 1. Calibration of the biosensor

We measured the luciferase activity of the p-sdh-lux plasmid during growth in a microplate reader (37°C with shaking). Each well contained 180 μl of minimal medium (M9) supplemented with glucose (0.3%) and the indicated concentration (0, 5, 10, 25, 100, 1000 μM) of cAMP. We added 20 μl of a LB pre-culture (*cyaA*- strain transformed with the p-sdh-lux luminescence biosensor) to each well at time zero. The promoter activity, computed from luminescence data, was calculated from the expression profile after the glucose exhaustion. The minimal detectable cAMP concentration of this biosensor, corresponding to a measurable difference in promoter activity, is around 5-10 μM.

2 Stochastic simulation of the communication

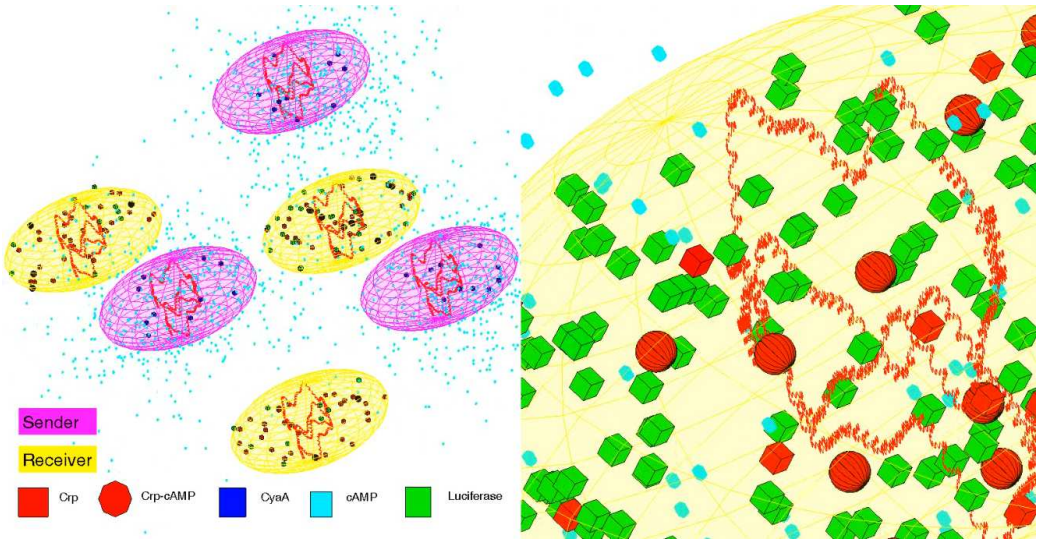

Figure 2. Supplementary movie 2.

We have implemented a stochastic, three-dimensional model to simulate the communication process. The code has been written in Matlab and is available upon request. Although this model reproduces the observed patterns, it should rather be considered as didactic. We do not derive any parameters from this simulation.

We added the video in flash format in this pdf document. Movie 2 shows the same simulation at a higher resolution. The screen of the video is divided in two parts. On the left, the video schematizes the different steps of the communication process. On the right, camera rotations and zooms highlight the tridimensional space and the events that we describe below. Each screen corresponds to a distinct simulation. Consequently, due to stochasticity in gene expression, the two simulations are not completely synchronous. At the beginning of the video, one can see three yellow ellipsoids that represent three *cyaA*-receiver cells. The three pink ellipsoids represent the *crp*- senders. All cells harbor a red, super-coiled plasmid. The sender cells produce adenylate cyclases (dark blue cube) that move randomly inside the cell and synthesize cAMP (light blue cube). Contrary to the proteins, cAMP progressively diffuses into the external medium, enters the receiver cells and binds to Crp proteins to form a cAMP-CRP complex. The molecular interactions, too computationally expensive, are not modeled. When cAMP molecules exceed a certain threshold in the receiver cell, there is a certain probability that CRP changes its conformation

(cube to sphere), increasing its affinity for binding site on the plasmid. Once bound to the DNA (watch the event on the right screen), the synthesis rate of luciferase increases, allowing transcription and traduction of *luxCDABE* and subsequent appearance of luciferase molecules (green cube) in the receiver cells. This event signals the success of the communication procedure. The receiver cells become green at different time due to stochasticity in gene expression. Obviously, those temporal differences can not be observed in our experimental data (presented in figure 2), obtained at the population level.

3 Investigation of the mouse gastro-intestinal ecosystem

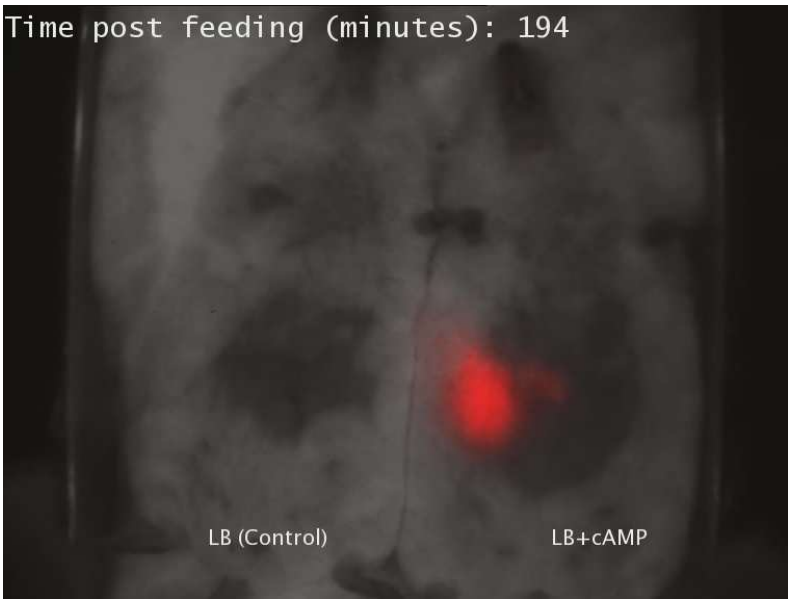

Figure 3. Supplementary movie 4.

We wanted to test whether communication by way of extracellular cAMP could occur between *E. coli* and other species in their natural environment: the gastro-intestinal tract. Unfortunately, we could not answer this particular question. The experiment shown here nevertheless demonstrates that external cAMP can induce gene expression in *E. coli* within the gastro-intestinal tract. It has previously been shown that the light produced by an *E. coli* population that has invaded the intestine of a mouse can be detected "transcutaneously" by a sensitive CCD camera. In our experiment, we administered orogastrically 200 μl of a solution containing approximately 25 billion *cyaA*- bacteria (containing the luminescence biosensor) to two mice. After 30 minutes, we administered orogastrically 250 μl of LB containing 100 mM cAMP to one mouse and 250 μl of LB to the second mouse (control). We then anesthetized the mice and placed them in a dark chamber under a sensitive camera to acquire luminescence images. Movie 4 shows that the expression of a gene (*luxCDABE*) is switched on in the gastro-intestinal tract of the mouse that has received cAMP. The mouse on the right, that drank the LB + cAMP solution, emits a strong luminescence detected transcutaneously and thus in a non invasive way. The mouse on the left, that drank LB as control, produces no light. We have reproduced this experiment twice.

Materials and methods

Briefly, two Swiss CD-1 mice (Janvier, Le Genest-Saint-Isle, France) were given drinking water containing 500 μg/ml of kanamycin and ampicillin for 16 h to kill most commensal bacteria. The *cyaA* strain containing luminescence and fluorescence biosensors was grown in 100 ml of LB medium (with 100 μg/ml ampicillin and 50 μg/ml kanamcyin) overnight at 37°C under shaking. We resuspended the cells in 2 ml of LB and we administered orogastrically 200 μl of this solution with a feeding needle. 30 minutes later, we administered 250 μl of LB 100 mM cAMP to one mouse and 250 μl of LB to the second mouse (control). The mice were then anesthetized with a mixture of ketamine hydrochloride (Imalgen 500, Merial, 25 mg/kg body weight) and xylasine (Rompun, Bayer Healthcare, 12.5 mg/kg body weight), injected intra-peritoneally . The two mice were placed in a heated dark chamber and filmed for several hours by a sensitive CCD camera. Luminescence images were acquired every 30 seconds. We had to anesthetize the mice three times during the kinetics. This explains why the luminescence spot moves in the film just before the next anesthesia: a mouse begins to awake. The images of mice do not detect the awakening because there are static images taken at the beginning of each anesthesia. These images were subsequently overlaid onto the luminescence images (red false color). The movie was created using Matlab. No specific image enhancement has been performed.

Ethical statement

Animals were housed according with the French Ethical Committee (Decree 87-848) and European Community Directive 86/609/EEC. Experiments were carried out under the supervision of CL (agreement nu 38 08 08) in the animal care facilities approved by the Direction des Services Vétérinaires de l'Isère (Nu A 38 516 10006).

Chapitre V

Using luciferase activity to infer the metabolic state of *Escherichia coli*

Using luciferase activity to infer the metabolic state of *Escherichia coli*

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Abstract

The terrestrial luciferase of *Photorhabdus luminescens*, encoded by the *luxCDABE* operon, is a classic reporter system used for monitoring promoter activity. In order to emit photons, the luciferase requires an energy source provided by a functioning central metabolism. When the cell lacks energy, there is an Abrupt Decline of Luciferase Activity (ADLA). In this manuscript we show that this dependence of luciferase activity on the availability of cellular energy sources can be used to infer metabolic states of the cell *in vivo* and in real time. The system can also be used to measure more specific parameters such as the relative activity of a specific enzyme, the speed of acetate consumption, the overflow metabolism or the level of catabolite repression. Combined with a library of mutants, the measurement of luciferase activity may also help to increase the accuracy of our metabolic maps or provided a tool for similar applications.

Introduction

Characterization of the response of *Escherichia coli* cells to genetic and environmental perturbations is often performed *ex situ* using fast sampling techniques with or without a quenching step. Analyses of the transcriptome, proteome and/or metabolome are carried out *a posteriori*, after cell disruption, using DNA microarray or mass spectrometry (Ishii *et al.*, 2007). Those powerful, high-throughput technologies provide large amounts of data at the genome scale, but they do not give access to the behavior of the organism in real time.

Conversely, GFP and luciferase reporters allow measuring promoter activities in living cells (Hakkila

et al., 2002; Van Dyk *et al.*, 2001) with a high temporal resolution (Zaslaver *et al.*, 2006). Contrary to GFP, which needs an external input (excitation light) to produce the output signal, bacterial luciferase emits photons without external dependencies. The ease and sensitivity of light detection, associated with the absence of background, has led to numerous applications using luciferase, such as enzymatic assays, biosensors and *in vivo* imaging (Waidmann *et al.*, 2011).

The terrestrial luciferase of *Photorhabdus luminescens* is part of a five genes operon (*luxCDABE*). The genes of the operon code for luciferase as well as the enzymes necessary for producing the substrates of the light generating reaction. The key substrate, a long-chain aldehyde, is produced within the cell by the reduction of fatty acid catalyzed by the gene products of *luxC, luxD,* and *luxE*. The hetero-dimeric luciferase, encoded by *luxA* and *luxB* genes, oxidizes this substrate and $FMNH_2$ in presence of oxygen, to emit a photon at 490 nm (Meighen, 1991). The reaction is as follow:

$$FMNH_2 + RCHO + 0_2 \rightarrow FMN + H_20 + RCOOH + h\nu \text{ (490 nm)}$$

The production of $FMNH_2$ requires an electron transport system, driven by the central reactions of carbon and energy metabolism (Sunya *et al.*, 2011). Consequently, light production does not only reflect the quantity of luciferase, but also the concentration of the metabolites participating in the light-generating reaction, and thus the activity of the metabolic reactions that produce these metabolites. For example, it has been shown that an Abrupt Decline of Luciferase Activity (ADLA) occurs upon entry in stationary phase of *Escherichia coli* cells due to a drop of the intracellular redox pool (Koga *et al.*, 2005).

In this study, we present several ways in which ADLA, and, more generally the variation of luciferase activity, can be exploited as a measure of the metabolic state of the cell. The variation of luciferase activity, combined with the appropriate genetic tools (mutants), allows to assess specific metabolic fluxes and thus indirectly monitor the activity of the corresponding enzymes.

Results

Detection and characterization of the abrupt decline of luciferase activity

We first wanted to show that ADLA can be used as a measure for the availability of a carbon or energy source. In order to verify this basic premise, we grew *Escherichia coli* expressing the *luxCDABE* operon from *Photorhabdus luminescens* on a minimal medium with glucose as a carbon and energy source. We

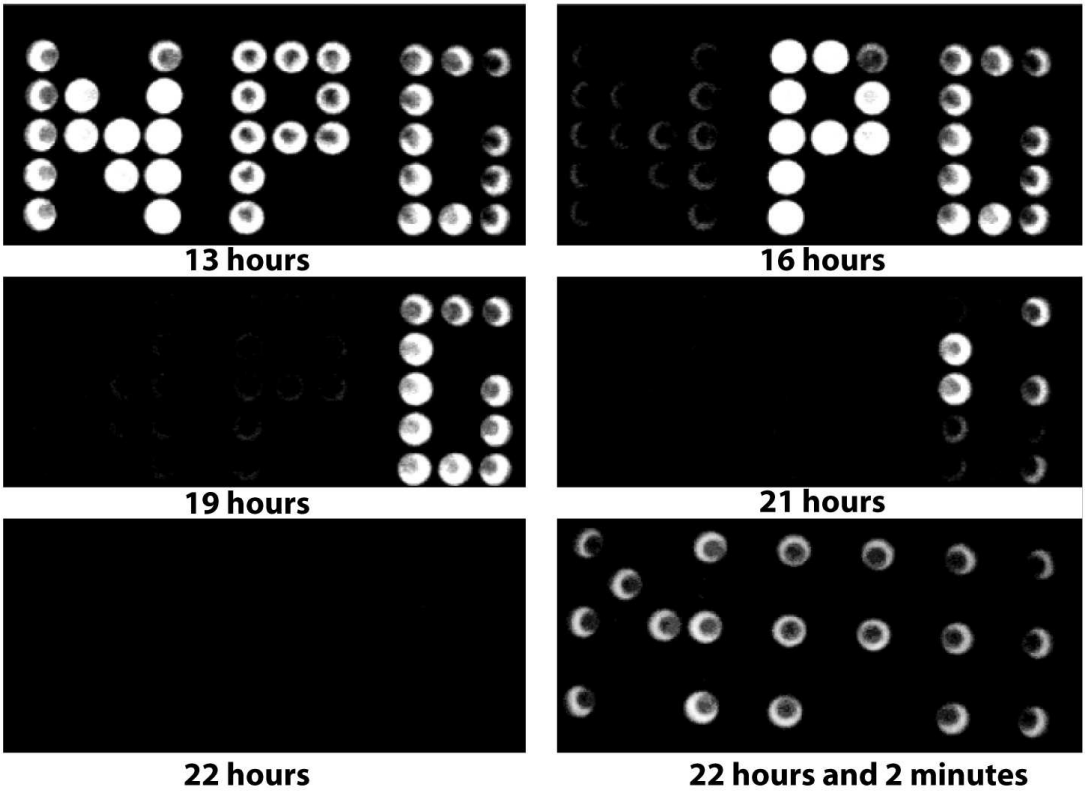

Figure 1. The abrupt decline of luciferase activity depends on the energy supply of the cell. Luminescence images extracted from supplementary movie (see details in supplementary information). Specific wells of a 96-well microplate (forming the letters N, P, G) contain a minimal glucose medium and *Escherichia coli* cells expressing the *luxCDABE* operon. Glucose concentration of each letter increases from left to right on the microplate (0.01% to 0.06%). Glucose consumption thus takes more time for cells further to the right on the plate. When the external energy source is exhausted, the energy production of cells drops and letters of this "bacterial billboard" switch off one after the other. Adding glucose to the medium (here in one well out of two) immediately turns on the luminescence signal again, demonstrating that luciferase enzymes are still present within the cells.

observed light production by the luciferase during the growth phase and an abrupt extinction of light emission when the energy source was exhausted. To illustrate the phenomenon, we created a bacterial billboard by growing the bacteria in a 96-well microplate with different amounts of glucose in the different wells (Figure 1 and supplementary information: movie). The concentration of glucose increases from left to right in the microplate. As soon as energy production stops (the duration of growth is a function of the glucose concentration), luciferase activity drops sharply. The luciferase enzymes are still present after the cessation of light production, since adding glucose after the ADLA allows immediate recovery of the original luminescence signal.

Measuring the metabolic state of cells

Not surprisingly, this experiments shows that light emission is completely shut off when cellular energy is exhausted. Could we obtain a more graded response and use the intensity of light emission as a measure of the intracellular energy level ? In order to separate signals controlling light production, i.e., the regulation of *luxCDABE* transcription, and the energy requirement of luciferase activity, we use the synthetic *tet* promoter to drive the expression of the *luxCDABE* operon. The activity of this promoter is repressed by the TetR repressor, and repression is lifted by adding anhydrotetracycline (aTc) to the medium. We grew a wild-type strain containing this construction on a minimal medium containing glucose, acetate and the inducer (aTc). The temperature was maintained at 37°C and we measured the absorbance and luminescence of the culture grown directly in a microplate reader. The presence of the inducer from the beginning of the kinetics directly triggers the transcription of *luxCDABE* (Figure 2A). When glucose is exhausted (break on the red curve), there is a sharp decrease immediately followed by a rapid recovery of the luminescence signal. This event marks the transient fall of energy during the glucose-acetate transition. When acetate is exhausted, there is no remaining energy source in the medium and we observe an abrupt decline of luciferase activity (ADLA). Thus, measuring luciferase activity driven by a constitutive promoter during the growth of the bacterial culture can be used to detect, in real time, small and large variations of energy supply.

Since the luminescence profile gives an indication of energy availability, we can use this signal to assess the effect of different mutants on the energy metabolism of *E. coli*. We therefore transformed hundreds of single-gene knock-out mutants (Baba *et al.*, 2006) with the biosensor plasmid and measured their corresponding luminescence profile in the same conditions as in Figure 2A. Most of the mutants

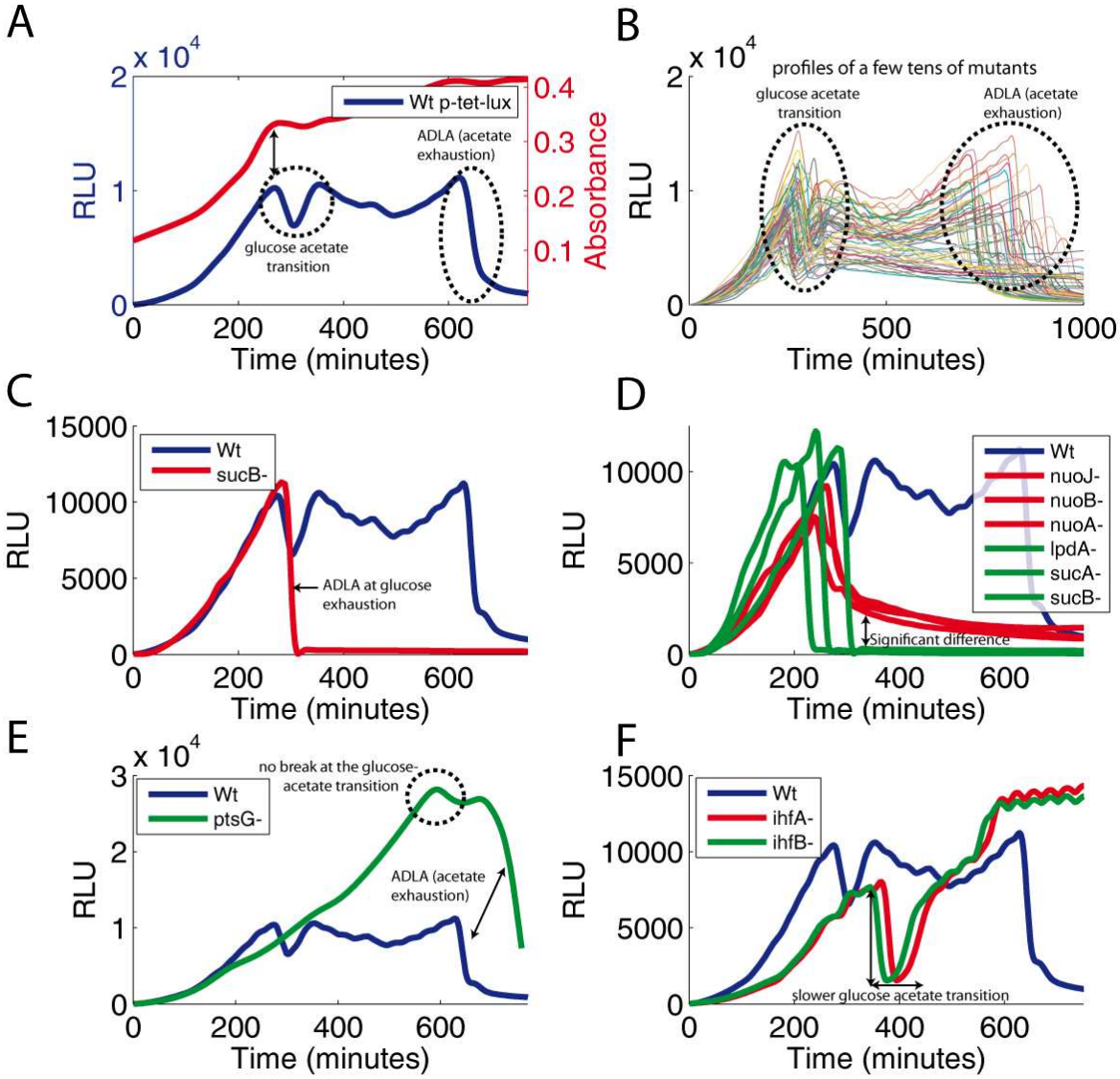

Figure 2. Luciferase activity during growth on glucose and acetate in different genetic backgrounds. (A) An *E. coli* wild-type strain, transformed with the *p-tet-lux* plasmid, grows on a minimal medium with glucose (0.03%), acetate (0.03%) and aTc (500ng/ml). When glucose is exhausted, there is a sharp decrease followed by a rapid recovery of luciferase activity. This occurs exactly at the time when growth slows, i.e., when glucose is exhausted (arrow). The Abrupt Decline of Luciferase Activity (ADLA) signals acetate exhaustion. (B) Example of few dozens of mutants showing a profile similar to the one of the wild-type strain. Difference in the time delay of ADLA may therefore reflect difference in the speed of acetate consumption. (C) The profile of a *sucB* mutant shows an ADLA when glucose is exhausted because this strain can not utilize acetate as an energy source. (D) During growth on acetate, *nuo* mutants maintain an intermediate level of luciferase activity. (E) In a *ptsG* mutant, there is no transient decrease of light emission at the point of glucose exhaustion. (F) The more pronounced dip in luciferase activity in *ihfA* and *ihfB* profiles may indicate a slower glucose-acetate transition.

have a luminescence profile similar to that of the wild-type strain (Figure 2B).

However, a subset of the mutants (*sucB, sucA, lpdA, atpC, atpD*) fails to maintain luciferase activity after glucose exhaustion (Figure 2C; exemplified by the *sucB* mutant). In fact, the oxoglutarate dehydrogenase complex and ATP synthetase are needed for growth on acetate. In these mutants, the impossibility to use acetate as an energy source after glucose exhaustion directly leads to the abrupt decline of luciferase activity. For mutants that are impaired in using acetate as an energy source, we observe intermediate phenotypes. The *nuo* operon shown in figure 2D is an example. We interpret the different levels of light production by these mutants as a measure of metabolic flux. The small differences of the growth phenotype would be impossible to detect and a full blown metabolic analysis would be very time consuming and costly. Consequently, we propose to use the capability to maintain luciferase activity as a very sensitive measure of the metabolic state of the cell.

Certain mutants show interesting phenotypes, but are more difficult to interpret. The *ptsG* mutant lacking the main glucose transporter takes much more time to consume glucose, as expected. However, we did not observe the transient decrease of the luminescence signal when glucose is exhausted (Figure 2E; locate the circle). The glucose-acetate transition may occur without transient drop of energy or the cells may consume glucose and acetate at the same time during growth. On the contrary, the transient drop of light emission at the glucose-acetate transition is much more pronounced in the *ihfA* and *ihfB* mutants, compared to the wild-type strain (Figure 2F). This phenotype may reflect a slower glucose-acetate transition in the IHF deficient strain. IHF is a transcriptional activator of the *aceBAK* operon, which codes for enzymes of the glyoxylate by-pass (Resnik *et al.*, 1996), the crucial pathway for gluconeogenesis and thus for acetate utilization. The absence of IHF may slow the establishment of this pathway, an event reported by the almost complete, transient loss of luciferase activity after glucose exhaustion.

Tuning and measuring acetyl CoA synthetase activity *in vivo*

The transient decrease in luciferase activity after glucose exhaustion probably represents the time needed by the cell to switch from glucose metabolism to the utilization of acetate, the only remaining carbon source. The key enzyme for acetate utilization is acetyl-CoA synthetase, coded by the *acs* gene. This enzyme converts acetate to acetyl coenzyme A, which is the first enzymatic reaction allowing acetate utilization. By controlling the expression of *acs*, we should therefore be able to control the luciferase

activity of a "metabolic biosensor".

acs transcription is activated by the Crp-cAMP complex after glucose exhaustion and there is no promoter activity at all in a mutant lacking adenylate cyclase, the enzyme producing cAMP coded by the *cyaA* gene. However, this lack of cAMP can be complemented by adding exogenous cAMP to the growth medium. Different concentrations of added cAMP activate the *acs* promoter to different extents. If our hypothesis is correct, this should lead to different levels of acetyl-CoA synthetase leading to different levels of energy in the cell, which could be detected by the appropriate biosensor. Figure 3A shows the expression of *acs* in a *cyaA* strain, detected by a fusion of luciferase to the *acs* promoter. The transcriptional response is clearly dependent on the concentration of cAMP in the growth medium. The promoter is shut-off during growth on glucose. The *p-acs-lux* fusion thus reports essentially *acs* transcription and not energy availability of the cell.

In order to measure luciferase activity as a function of energy supply, we used the *aceBAK* promoter, again fused to the luciferase operon (*p-aceBAK-lux*). The very strong *aceBAK* promoter leads to a very high luciferase concentration. At those levels of luciferase activity, variation of light emission mainly reports changes in the energy supply rather than the regulation of the *aceBAK* promoter. Consequently, in our experiment, the *aceBAK* biosensor mainly reports the acetyl-coenzyme A synthetase activity, i.e., the rate of acetate metabolism.

We grew the *cyaA* cells as before on minimal medium in the presence of glucose and acetate, but without exogenous cAMP. The *aceBAK* promoter is highly transcribed during growth on glucose (Figure 3B, times before the red arrow). Luciferase activity drops sharply when glucose is exhausted (red arrow). In this state, the *cyaA* mutants no longer grow because acetyl-CoA synthetase is not produced. We kept the cells in this state for about an hour, then added different concentrations of cAMP to the medium. This addition triggers the activation of *acs* transcription (Figure 3A). The arrival of acetyl coenzyme A synthetase enzymes triggers utilization of acetate as a carbon source and thus allows the recovery of luciferase activity of the *aceBAK* biosensor (Figure 3B). The luciferase activity reflects energy production and thus the rate of acetate consumption which is function of the concentration of acetyl coenzyme A synthetase. Indeed, the luciferase activity of the *aceBAK* biosensor during growth on acetate (Figure 3B) correlates well with the activity of the *acs* promoter (Figure 3A). Furthermore, faster acetate utilization (at higher cAMP concentrations) also leads to an earlier onset of ADLA (Figure 3B, blue arrows), because the fixed amount of acetate in the medium is consumed more rapidly.

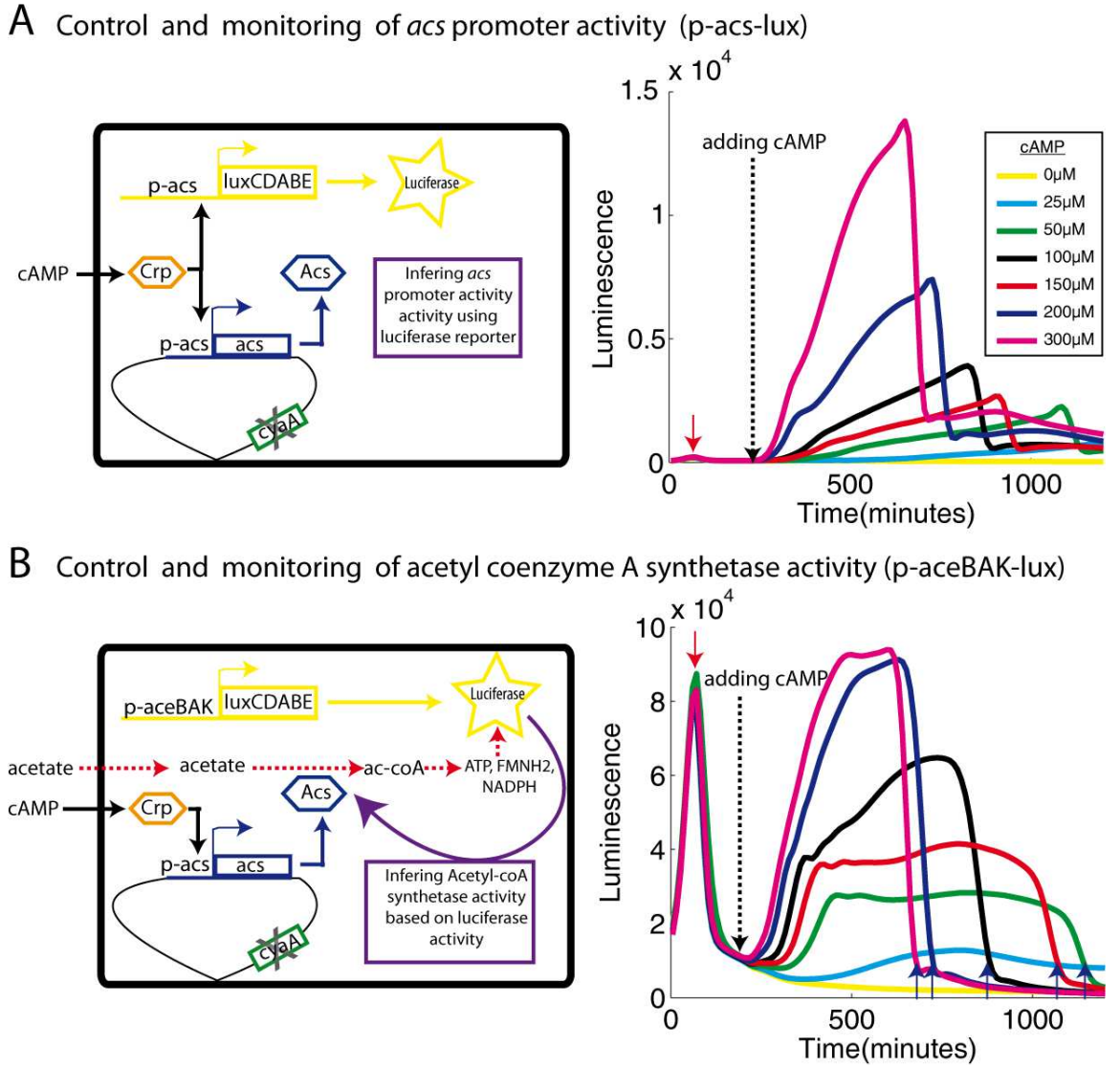

Figure 3. Inferring the activity of a specific enzyme by measuring luciferase activity. The *cyaA* strains were grown on a minimal medium supplemented with glucose (0.015%) and acetate (0.03%). We added different concentrations of cAMP to the medium at 200 minutes. (A) The *cyaA* mutant transformed with the *p-acs-lux* plasmid. Luciferase activity mainly reports the transcription of the *acs* gene, which is activated by the added exogenous cAMP in a dose dependent manner. (B) The *cyaA* mutant transformed with the *p-aceBAK-lux* plasmid. The light emission during growth on glucose reaches a high level (red arrow). After glucose exhaustion, there is an abrupt decline of luciferase activity since the *cyaA* mutant can not grow on acetate. Adding cAMP allows *acs* transcription, production of acetyl coenzyme A synthetase, and recovery of luciferase activity in a dose dependent manner. The ADLA occurs more quickly when the acetyl coenzyme A synthetase concentration is high because acetate is consumed more rapidly (blue arrows).

To conclude, in this experiment, we monitored, *in vivo* and in real time, the relative level of acetyl coenzyme A synthetase and thus the rate of acetate consumption by using the information provided by the recovery of luciferase activity. We checked our interpretation by using a *cyaA acs* double mutant that fails to recover luciferase activity despite the presence of exogenous cAMP, as expected (see supplementary information).

Correlation between ADLA and the end of glucose repression

We have shown in a previous work that carbon catabolite repression (CCR) senses the metabolic state of the cell, probably energy availability or glycolytic flux. Using our biosensor that responds to energy availability, we can assess the connection between this parameter and the expresssion of CCR-controlled promoters.

To monitor CCR, we use the *araBAD* promoter. This promoter is activated both by the AraC-arabinose complex and by the Crp-cAMP complex. Since we wanted to study only CCR, we added the inducer (arabinose) to all media in these experiments. As expected, in these conditions, we observe that the expression of *araBAD* is strongly induced when glucose is exhausted.

To assess energy availability and CCR in the same cell, we transformed a *sucB* mutant with two biosensors (Figure 4A): the previously described p-tet-lux plasmid that reports the energy level of the cells (luminescence), and the p-araBAD-gfp plasmid, that measures the activity of the *araBAD* promoter (fluorescence). We grew the strain in a minimal medium containing glucose and the inducers (arabinose and aTc). As expected for a *sucB* mutant, we observe an abrupt decline of luciferase activity once glucose exhaustion (Figure 4B). Simultaneously with ADLA (luminescence), we observe the induction of the *araBAD* promoter (fluorescence) (Figure 4B vs 4C; black dashed arrow). The drop of luciferase activity indicates the decrease in energy availability and this event seems to trigger the transcription of cAMP-CRP controlled promoters. We confirmed this result with another Crp-cAMP dependent promoter (*acs* promoter; data not shown). When all energy sources are used up, no residual luciferase activity remains (Figure 4BC, red dashed arrow) and the transcription of *gfp* also stops. All cellular functions seem to be shut down at this point.

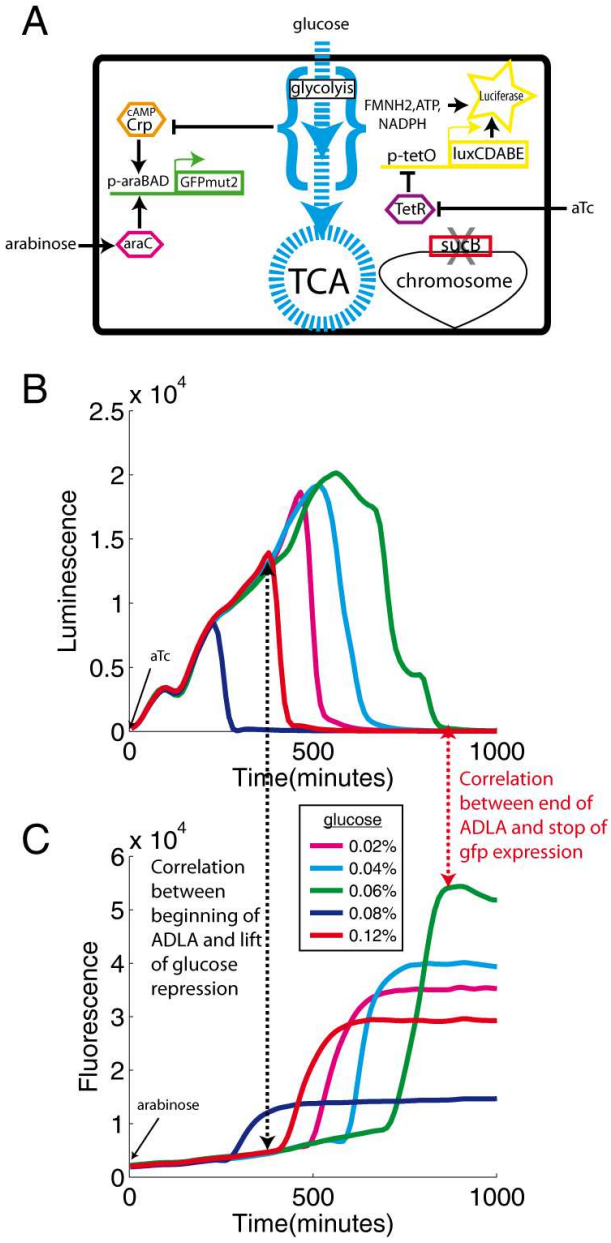

Figure 4. Measuring carbon catabolite repression. (A) A *sucB* mutant was transformed with two plasmids. The first plasmid, *p-araBAD-gfp*, reports *araBAD* promoter activity (fluorescence, C) and the second plasmid, *p-tet-lux*, monitors the energy level of the cell (luminescence, B). The strain grew on a minimal medium supplemented with glucose, arabinose (0.005%), and aTc (500ng/ml). (B) Profile of luminescence. ADLA occurs once glucose is exhausted (in a dose-dependent manner) (C) Induction of *araBAD* occurs simultaneously with the beginning of the ADLA (C vs B). The arrest of gfp expression (fluorescence) occurs simultaneously with the end of the ADLA.

Quantifying the overflow metabolism

In addition to measuring intracellular physiological states, we can use the reporter system also to assess the media composition. As described above, *acs* expression is strongly induced when glucose is exhausted. The *p-acs-lux* plasmid can therefore be used to detect glucose exhaustion. By varying the concentration of glucose, in the presence of a fixed acetate concentration, we observe a strong increase of luminescence correlated with the glucose concentration (Figure 5A). Conversely, by fixing the glucose concentration and varying the acetate concentration, we can control the time of the abrupt decline of luciferase activity that marks acetate exhaustion (Figure 5B). Combining these two phenotypes, we can use the *p-acs-lux* plasmid to detect both glucose and acetate exhaustion in real-time.

The transition from growth on glucose to growth on acetate is a natural phenomenon for *Escherichia coli*. When these bacteria grow rapidly on glucose, they excrete acetate in the culture medium (Wolfe, 2005). This phenomenon have been called "overflow metabolism". Once glucose is exhausted, bacteria import the secreted acetate and used it as a carbon source. Using our reporter plasmid, we were able to quantify the overflow metabolism. We grew a wild type strain carrying the *p-acs-lux* plasmid in varying concentrations of glucose, but without adding acetate to the medium. With increasing glucose concentration, we observe a proportional increase of the delay between *acs* induction (reporting glucose exhaustion) and ADLA (reporting acetate exhaustion) (Figure 5C). We believe that this delay is indicative of the amount of acetate secreted due to overflow metabolism.

An easy way to test this hypothesis consists in testing mutants known to limit overflow metabolism and acetate secretion due to low glycolytic flux (Lin *et al.*, 2005). Compared to a wild-type strain, both the *ptsG* mutant (that lacks the main glucose transporter) and the *pgi* mutant (that interrupts glycolysis) show a delayed *acs* induction. This is expected for strains that consume glucose more slowly (Figure 5D). However, for the same glucose concentration, the delay between *acs* induction and the ADLA is much shorter in these two strains compared to the wild type strain. Our *p-acs-lux* biosensor is therefore a simple tool for estimating acetate secretion and overflow metabolism.

Discussion

In this study, we have shown that bacterial luciferase can be used, both as a reporter of promoter activity and of the metabolic state of the cell. When there is excess energy, the reporter system measures

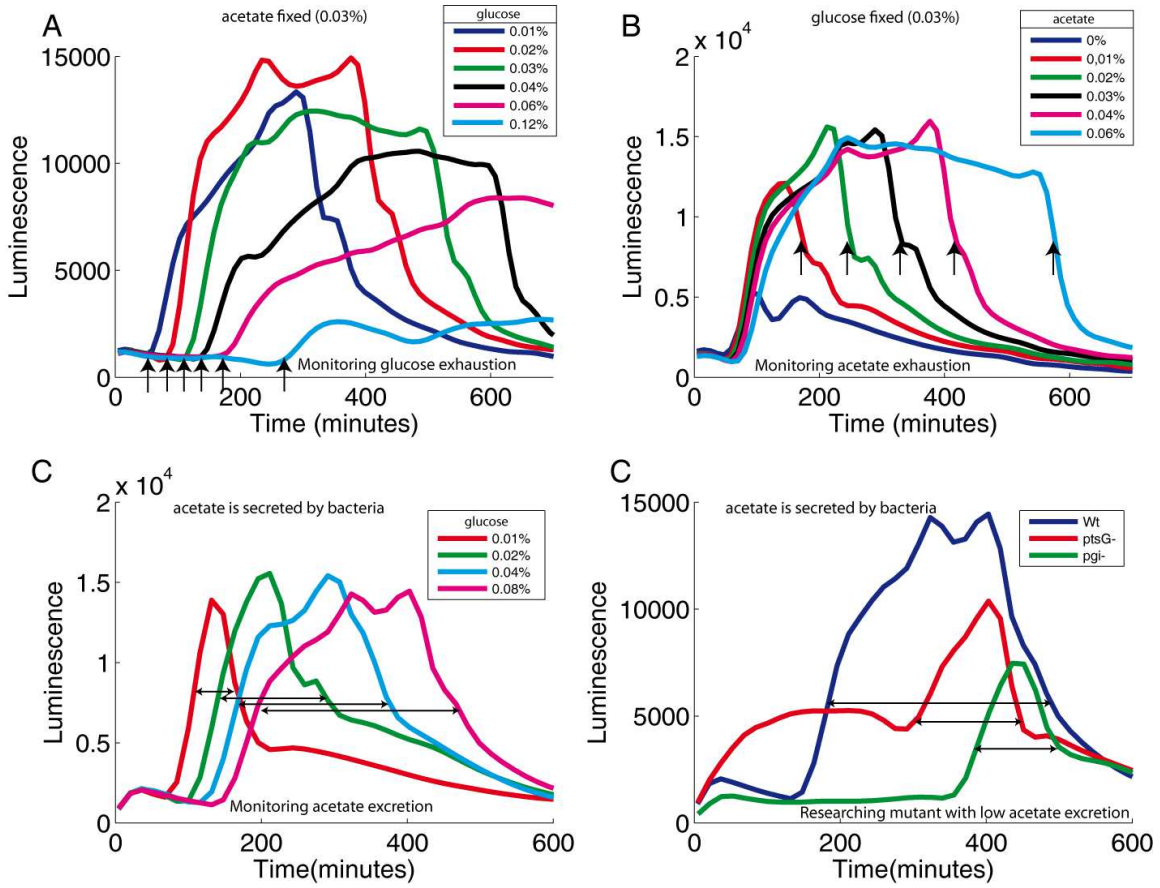

Figure 5. Measuring glucose, acetate, and overflow metabolism. (A) The *p-acs-lux* plasmid can be used to monitor glucose exhaustion (acetate concentration is fixed). A strong increase of luminescence occurs upon glucose exhaustion (in a dose dependent manner). (B) The *p-acs-lux* plasmid can also be used to monitor acetate exhaustion (glucose concentration is fixed). The time of Abrupt Decline of Luciferase Activity (ADLA) is proportional to the acetate concentration. (C). Bacteria were grown on a minimal medium with glucose, but without acetate. When the glucose concentration increases, the delay between *acs* induction (glucose exhaustion) and ADLA (acetate exhaustion) increases proportionally. This delay indicates the amount of secreted acetate during growth on glucose due to overflow metabolism. (D) For the same amount of glucose (0.08%), mutants with a reduced glycolytic flux (*ptsG, pgi*) show lower acetate secretion compare to the wild-type strain.

transcription, when cellular energy is limiting, the luciferase activity is determined by the metabolic state of the cell and the reporter system measures metabolic activity. The schematic of figure 6A and the following paragraphs summarize the main results.

Global information

The main parameter to consider in order to measure the global metabolic activity of the cell is the ratio luciferase-activity / luciferase (ratio LA/L), especially when luciferase concentration (L) is high. When the activity of metabolism, or cellular energy, drops, this ratio drops. The accuracy of light detection, and its large dynamical range, allow detecting small variations of energy supply.

The ratio LA/L also allows to estimate the functioning of the global expression machinery of the cell in a rather obvious way: when no energy is available at all, the luciferase activity, and therefore the ratio LA/L, drops to zero. In synthetic biology, regulatory networks are sometimes considered independently of the activity of metabolism. Yet, variations of the metabolic activity have a profound effect on gene expression and the regulation of gene expression. The LA/L ratio may provide a useful parameter to assess the capacity of the cell to perform the function of a designed, or natural, genetic regulatory network. Last, at intermediate levels of the LA/L ratio, we can assess the activity of certain Crp-cAMP dependent promoters.

Specific information

Depending on the experimental condition, the LA/L can also be used to obtain more specific information. In this study, we have used this parameter to quantify the activity of acetyl coenzyme A synthetase (and the corresponding flux of acetate utilization). By controlling the expression of acetyl coenzyme synthetase, our *aceBAK* biosensor correctly indicated the rate of acetate utilization and thus the degree of energy availability of the cell. This method of detection/relative quantification of enzyme activity is not limited to acetyl coenzyme A synthetase. The activity of any enzyme could be quantified by setting the conditions such that this enzyme constitutes the bottleneck for luciferase activity. For instance, controlling *lacZ* expression in cells growing on a minimal medium with lactose as the only carbon source will limit the rate of metabolism and a luciferase-based biosensor of metabolic activity would thus indirectly measure β-galactosidase activity.

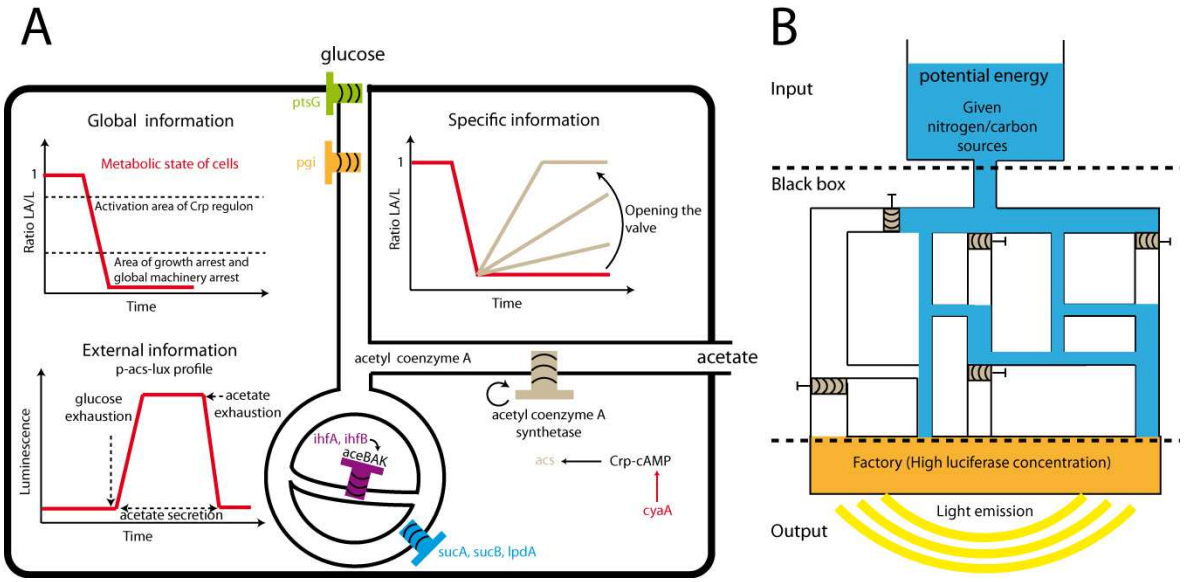

Figure 6. Schematic of the biosensors. (A) Representation of a cell. Metabolism (glycolysis and TCA) is represented by pipes, the opening of which can be controlled by valves (mutants). Valves that are shown on the pipes have been used in this work: *ptsG, pgi, ihfA, ihfB, sucA, sucB, lpdA*. The valve "acetyl-coenzyme A synthetase" is more complicated: the degree of opening can be adjusted by the level of cAMP, since cAMP-CRP controls *acs* transcription. The three schematics placed inside the cell show the different kinds of information that can be assessed using luminescence reporters. Specific information (top right) can be obtained by measuring the ratio "luciferase activity/luciferase" (LA/L) in controlled conditions (adjustment of the valve opening). Global information (top left) can also be obtained: a very low LA/L ratio indicates a global shut-down of cellular functions. Intermediate LA/L ratios indicate a regime where targets of the Crp regulon are activated. External information (bottom left) can be inferred using the *p-acs-lux* transcriptional fusion: glucose exhaustion is detected by the induction of *acs* expression. Acetate exhaustion is detected by ADLA. The combination of these two pieces of information allows determining the amount of secreted acetate. (B) The energy is seen as a reservoir flowing in a network of pipes and operating an electric power station (luciferase), placed at the bottom and producing light. To determine the network topology and the flow, it is possible to adjust valves in the network (mutation) while watching the consequences on light production.

Information about media composition

In this study, we also present a biosensor, *p-acs-lux*, that responds to glucose exhaustion (via a transcriptional activation) and on acetate exhaustion (via the drop of metabolic activity). Taken together, those two signals can be used to measure acetate secretion during overflow metabolism. This biosensor could thus be useful, for example, in a screening procedure that aims at identifying mutants with low acetate secretion. The accumulation of acetate in the culture medium is an important problem in industrial fermentations since this organic acid inhibits cell growth and recombinant protein production. Different approaches are currently developed to reduce acetate accumulation by modifying central metabolic pathways (Gosset, 2005).

Redrawing metabolic flux

By combining the complete mutant library of *E. coli* with the current knowledge of metabolism, the ratio LA/L may help to re-draw, with a high resolution, the map of metabolic flux for a given medium. Indeed luciferase activity is particularly well-suited for detecting small metabolic leaks. We propose an analogy with a Dam (Figure 6B). Potential energy (water) is stocked as carbon or nitrogen sources. The water flow passes through a complex circuit with valves placed on each channel. At the downstream end, a factory (high luciferase concentration) transforms the potential energy in light, provided sufficient flux arrives at the factory. To reconstruct an unknown circuit of channels, the experimenter would close certain valves (knock out mutant) or enlarges other (over expression) and analyses the effect on light production. A serious limitation of this approach is the fact that luciferase activity is affected by "global energy availability" : ATP, NADPH, NADH, $FMNH_2$. A mutant affecting specifically the concentration of one of those metabolites without affecting the other could generate artifacts: the luminescence signal may drop even though the cell still has a lot of energy. However, among the dozens of mutants involved in energy production that we have tested, we did not find a single one (including *nuo*, *atp*, *fre*, *suc* mutants) growing normally without being able to maintain luciferase activity. In this sense, luciferase activity seems to be a robust, global measure of energy availability in the cell.

Conclusion

In this study we have shown that reporter systems based on bacterial luciferase may be used for inferring different parameters about the functioning of the cell. The ease and sensitivity of light detection, associated with its small cost, make our method/tool easy to implement/to use. The biosensors can potentially be used in many applications in biotechnology, system and synthetic biology.

Materials and Methods

Strains

The mutant strains come from the Keio collection (Baba *et al.*, 2006). The wild type strain (*E. coli* K-12 strain BW25113) has a well-defined pedigree and has not been subjected to mutagens (Datsenko & Wanner, 2000). We removed the kanamycin cassette of the *sucB* mutant using the flp recombinase to allow antibiotics compatibility. The *cyaA*- *acs*- double mutant was constructed by removing the kanamycin resistance cassette of the *cyaA*- strain. Then, we transduced (P1 phage transduction) the kanamycin resistance cassette of the *acs*- strain in the *cyaA*- strain. High-throughput transformation of hundreds of mutants was performed by using a previously described protocol (results not yet published).

Plasmids

We have used the p-acs-lux and p-tet-lux plasmids whose construction has already been described (results not yet published). The p-araBAD-gfp comes from the Uri Alon collection (Zaslaver *et al.*, 2006). The metA-aceB intergenic region was amplified by PCR using primers 5'-GCTGCTCGAGTCTTCTGTGAT AGTCGATC-3' (xho1 site underlined) and 5'-GTTCGAATTCCGTGCAGCTCCTCGTCAT-3' (EcoRI site underlined) using BW25113 strain as template. The PCR product, digested with Xho1 and EcoRI, was inserted into a *luxCDABE* plasmid backbone without promoter to make p-aceB-lux.

Measurement of light emission

Images of the figure 1 have been obtained by filming a microplate placed in a heated chamber using a sensitive CCD camera (photonic science). Histogram equalization has been performed to remove the luminescence background.

Dynamic measures of luminescence and/or fluorescence were performed by growing cells during several hours within a microplate reader (fusion alpha FP-HT). This micro plate reader allows growth with shaking and temperature control (37°C). It measure cell density (OD at 600nm), luminescence and/or fluorescence (485nm/520nm) at a high temporal resolution (5 minutes). Wells of the microplate contain 180μl of medium and 20μl (dilution 10) of cells in stationary phase. Cells in stationary phase (Figure 1, 2, 5) come directly from a LB pre-culture. Cells in stationary phase (Figure 3, 4) come from a LB pre-culture which have been centrifuged and the pellet have been re-suspended in a equivalent volume of clean M9 medium. The main parameters that differ between experiments are the strains, the concentrations of inducers (cAMP, arabinose and aTc) and the concentrations of the carbon sources (glucose and acetate). They are indicated in the legend of the figures.

Acknowledgments

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Supplementary information

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Video of ADLA

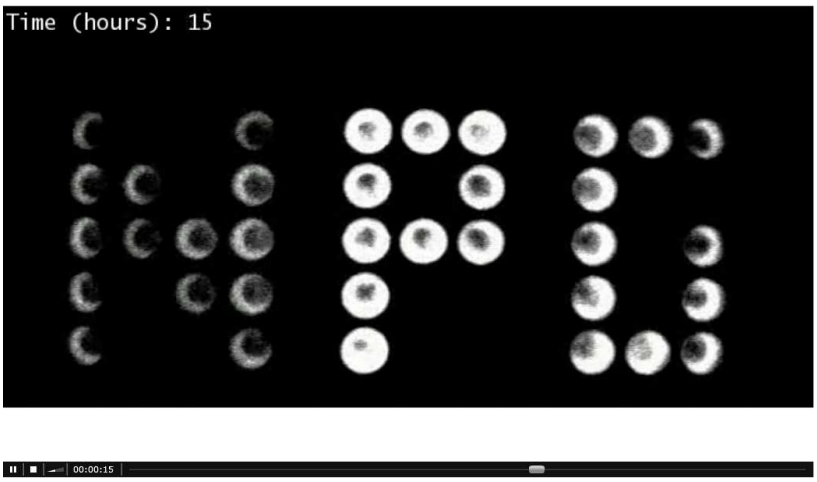

Figure 1. Supplementary movie.

Video from which were extracted the images of Figure 1. This bacterial billboard is obtained by filming for 25 hours the luminescence emitted by a 96-well microplate. The results obtained from this visualization of the cellular energy metabolism are somewhat more complete and complex than the description given in the results section. This video shows two different phenomena:

- carbon catabolite repression (CCR).
- Abrupt Decline of Luciferase Activity (ADLA).

The letters N, P, G were formed by appropriately grouping the wells of a 96-microplate. The wells forming the letters contained a minimal medium supplemented with glucose and acetate (0.03% w/v). The wells also contained the wild-type *E. coli* strain transformed with a reporter plasmid carrying the luciferase

operon (*luxCDABE*) under control of the *acs* promoter. The glucose concentration increases from left to right on the microplate (0.01% w/v to 0.06% w/v). Cells further to the right therefore grow for a longer time before exhausting glucose. When glucose is exhausted, the global regulator CRP turns on the transcription of the *luxCDABE* operon and letters switch on, one after the others. This effect is called catabolite repression.

At this stage, bacteria use acetate as a carbon source. When this nutrient is also exhausted, there is no remaining carbon source in the medium and the letters of the "bacterial billboard" switch off, one after the other. This effect is called Abrupt Decline of Luciferase Activity (ADLA).

Control of luciferase activity with a *cyaA- acs-* double mutant

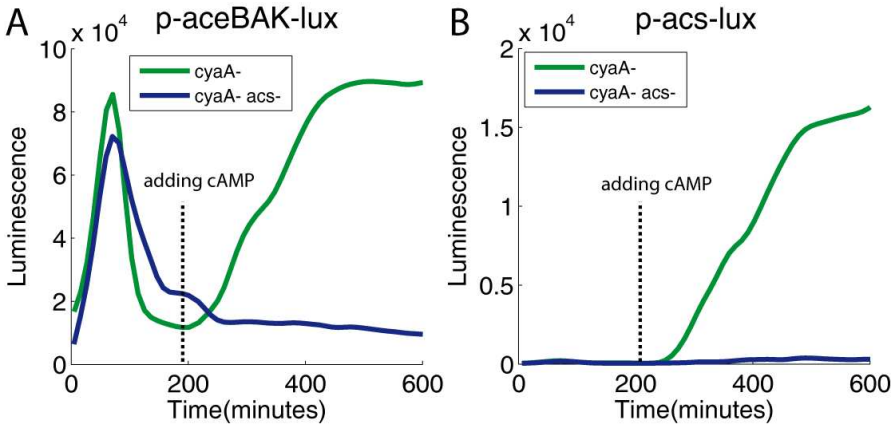

Figure 2. Control of luciferase activity with a *cyaA- acs-* double mutant. A *cyaA-* mutant and a *cyaA- acs-* double mutant were grown on minimal medium supplemented with glucose (0.015%) and acetate (0.03%). We added 5 mM cAMP to the medium at 200 minutes. (A) strains were transformed with the p-aceBAK-lux plasmid to monitor the energy level of the cell. There is a peak of luciferase during the phase of glucose consumption and the luminescence decreases when glucose is exhausted. In the *cyaA-* mutant, luciferase activity is restored by the addition of cAMP, which stimulates the transcription of *acs*, thereby allowing acetate to be used as a carbon source. In the *cyaA- acs-* double mutant, there is no recovery of luciferase activity. (B) The bacteria were transformed with the p-acs-lux plasmid (to monitor *acs* transcription): the transcription of *luxCDABE* operon from the *acs* promoter after adding cAMP only occurs if the chromosomal copy of *acs* is present.

In the experiment of the Figure 3B (see the section *Tuning and measuring acetyl CoA synthetase activity in vivo*), we have shown that adding exogenous cAMP in the growth medium leads to a recovery of luciferase activity. This phenomenon is probably due to the transcription of the *acs* gene, known to be induced by the Crp-cAMP complex. To definitively prove the essential role of this gene, we repeated this experiment in a *cyaA- acs-* background. In the supplementary figure 2A, we show that adding cAMP triggers the recovery of luciferase activity in the *cyaA-* mutant, but not in the *cyaA- acs-* double mutant, as expected. Moreover, in the *cyaA- acs-* double mutant, despite cAMP being added to the medium, we failed to induce *acs* expression (Figure 2B). This result strongly suggests that the production of acetyl-coA synthetase after the induction of the *acs* transcription is critical for allowing the consumption of acetate. This alternative carbon source then provides the energy source needed to continue transcription and/or translation of its own promoter. This positive metabolic feedback loop provides a physiologically reasonable regulation in adaptation to the environment: the production of the enzyme needed for utilizing acetate as a carbon source is stimulated by the action of this same enzyme.

Chapitre VI

Conclusion et perspectives

La plus grosse partie expérimentale de ma thèse aura consisté à essayer de comprendre pourquoi l'ajout d'AMPc exogène ne déclenche pas l'expression du gène *acs* quand il y a encore du glucose dans le milieu. Pourtant, après avoir testé des centaines d'hypothèses et criblé l'ensemble des simples mutants d'*E. coli*, nous n'avons pas pu déterminer le mécanisme à l'origine de ce résultat. Notre apport a donc principalement consisté à déconstruire les deux modèles principaux expliquant le phénomène de répression catabolique. Nous avons ainsi montré que la variation intracellulaire d' [AMPc] *ne peut pas* être à l'origine de la répression catabolique. Ce constat est d'autant plus paradoxal que l'AMPc joue neamoins un rôle de premier plan dans le processus. De même, nous avons montré que l'état de phosphorylation du PTS *ne peut pas* expliquer la répression catabolique malgré le fait que, encore une fois, le PTS y joue indéniablement un rôle. Nous proposons, humblement et sans certitude, de recentrer les recherches sur la protéine Crp et les variations de flux glycolytique (indépendamment du ratio PEP/pyruvate) : ce sont les deux seuls éléments qui, dans nos conditions, se sont avérés indispensables au fonctionnement de l'interrupteur. Une hypothèse potentielle proposée par Magasanik il y a maintenant 50ans (Magasanik, 1961) est celle du métabolite répresseur : peut-on imaginer un deuxième petit métabolite qui contrôlerait l'expression de Crp sans que nous nous en soyons rendu compte jusqu'à maintenant ? La solution réside-elle dans un mécanisme plus complexe, encore inconnu, et difficile à détecter avec les méthodes actuelles ? Ou, la solution nous est-elle tout simplement passée sous les yeux ? L'avenir nous le dira.

Ainsi, c'est sur ces recherches restées bloquées dans une impasse que se sont greffés les résultats qui suivent. Ces derniers sont plus sûrs, plus facilement reproductibles, plus intéressants

et débouchent *a priori* sur bien plus d'applications. Ils n'ont cependant nécessité qu'une petite fraction de mon temps de travail expérimental.

L'étude de l'opéron *araBAD* est un cas d'école pour décrire les variations stochastiques dans l'expression des gènes. Dans nos expériences, nous avons pu montrer qu'un flux glycolytique fort a tendance à synchroniser l'expression de l'opéron *araBAD* alors que la diminution de ce flux à l'aide d'un sucre non métabolisable module la distribution d'expression de cet operon dans la population de cellules. Les modélisateurs pourraient tenter de comprendre ce phénomène et inclure le flux glycolytique dans leur modèle stochastique.

Notre interrupteur métabolique, basé sur les activités inversées des promoteurs *acs* et *uhpT* en fonction du flux glycolytique, est très robuste, facile à utiliser et très rapide : l'inversion des expressions est visible en une dizaine de minutes. Il possède cependant deux défauts majeurs : on ne sait pas comment il fonctionne, car il est basé sur le mécanisme inconnu de la répression catabolique. De plus, il manque d'orthogonalité c'est-à-dire qu'il n'est pas du tout indépendant/découplé du réseau de régulation de la bactérie. Nous espérons cependant que notre système séduira des biologistes synthétiques ou des modélisateurs.

Un autre système synthétique développé dans cette thèse est le système de communication intercellulaire par l'AMPc. Ce système est robuste et facilement modulable, mais il souffre également du manque d'orthogonalité : il est directement connecté au « hub » que représente le complexe Crp-AMPc. Or, ce défaut *a priori* peut être transformé en avantage car ce complexe, très documenté dans la littérature, est déjà connecté à de nombreux modules (motilité, chimiotactisme, métabolisme, virulence, résistance au stress) et compose un grand nombre de motifs récurrents comme les boucles de type « feed forward ». Il existe, en outre, un très grand nombre de promoteurs contrôlés par le complexe Crp-AMPc et donc un large choix d'affinité de sites de liaison. Celui qui voudrait s'emparer de la difficile question de « la modularité » des réseaux de régulation génique pourrait donc trouver ici un terreau expérimental favorable.

Dans notre système de communication synthétique, il a fallu construire et optimiser deux souches. Une usine microbienne qui fabrique de fortes concentrations d'AMPc extracellulaire et un bio-senseur qui en détecte de très faibles. L'usine bactérienne est capable de produire plus de 150μM d'AMPc dans le milieu extérieur soit $\sim$10-100 fois plus qu'une souche sauvage. Cette concentration reste très éloignée des 30mM produit par Microbacterium sp. no. 205. Il est donc peu probable que notre souche soit compétitive à l'échelle industrielle. Cependant, ce rendement pourrait être amélioré en supprimant la phosphodiesterase (le gène *cpdA*) qui a justement un K_m de l'ordre de 500μM. Il n'est donc pas totalement exclu que l'amélioration de cette souche la rende compétitive. Le bio-senseur, lui, a sa limite basse de détection de l'AMPc extracellulaire vers 5-10μM. Or le K_m de l'AMPc pour Crp est dix fois plus faible. On sait

que l'export de l'AMPc est actif, mais le gène impliqué dans cet export n'a pas encore été découvert. Pour espérer « récupérer » ce facteur 10, il faudrait donc déterminer quels sont les gènes impliqués dans l'import / export de l'AMPc, puis il faudrait bloquer l'export et facilité l'import.

Il reste également à déterminer si le processus de communication intercellulaire par l'AMPc n'existe que dans les mains du biologiste synthétique ou s'il s'agit d'un mécanisme physiologique. Pourquoi la cellule gaspillerait-elle 9% de ses réserves en ATP à secréter de l'AMPc (Matin & Matin, 1982) ? Ce processus de communication, s'il il existe, n'est pas forcement « inter-bactérien » : il pourrait potentiellement être « inter-royaume », car l'AMPc est une molécule de signalisation utilisée également par les cellules eucaryotes. Nous avons entrepris des expériences visant à montrer un processus de communication entre nos cellules et l'amibe sociale *Dictyostelium discoideum*, sans succès néanmoins, faute de temps (Figure VI.1). Cependant, c'est aussi l'énorme avantage de notre petit système synthétique : il peut être implémenté entre des royaumes différents. Nous espérons donc que d'autres réussiront là où nous avons dû renoncer.

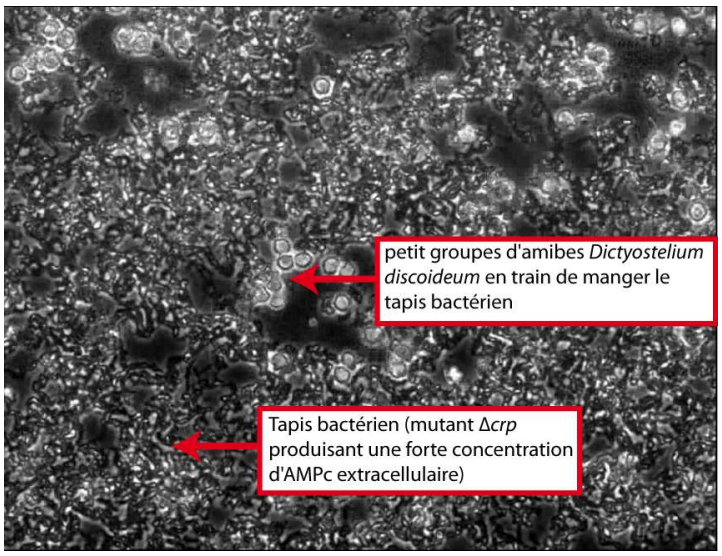

Figure VI.1 – Expérience pour tenter de faire communiquer deux royaumes distincts. Nous avons mélangé, sur un milieu solide, des bactéries Δ*crp* produisant de fortes concentrations d'AMPc extracellulaire avec des amibes sociales *Dictyostelium Discoideum* connues pour se regrouper grâce à l'AMPc. Cependant, il n'a pas été possible de tirer de conclusions concluantes sur ce type d'expériences.

Enfin, le milieu le plus logique où pourrait prendre place cette communication via l'AMPc chez *Escherichia coli* est l'écosystème gastro-intestinal. Les conditions y sont très différentes des conditions de laboratoire et la densité de cellules beaucoup plus forte pourrait faciliter un processus physiologique de communication. Pour tester cette hypothèse, nous avons inoculé à

des souris, par voie orale, la souche bio-senseur. L'idée était que le bio-senseur s'allumerait dans les intestins de la souris grâce à un processus de communication avec une souche « sender » physiologique. L'expérience n'a pas pu démontrer de processus de communication. Cependant, nous avons ensuite administré, par voie orale, de l'AMPc à la souris. Puis, nous avons observé un accroissement de lumière dans la zone du tractus gastro-intestinal et ce, de manière non-invasive, c'est-à-dire transcutané (Figure VI.2). Dans cette expérience, nous avons donc induit l'expression d'un gène (l'opéron *luxCDABE*) de bactéries placées dans le tractus gastro-intestinal d'une souris et nous avons contrôlé visuellement la réussite de l'induction. Ce type d'expériences pointe du doigt des technologies plus futuristes où nos intestins seront inspectées par des cellules médicaments, véritables micro-ordinateurs synthétiques, chargées de surveiller et de rétablir l'homéostasie de l'hôte ou de signaler la présence d'un pathogène.

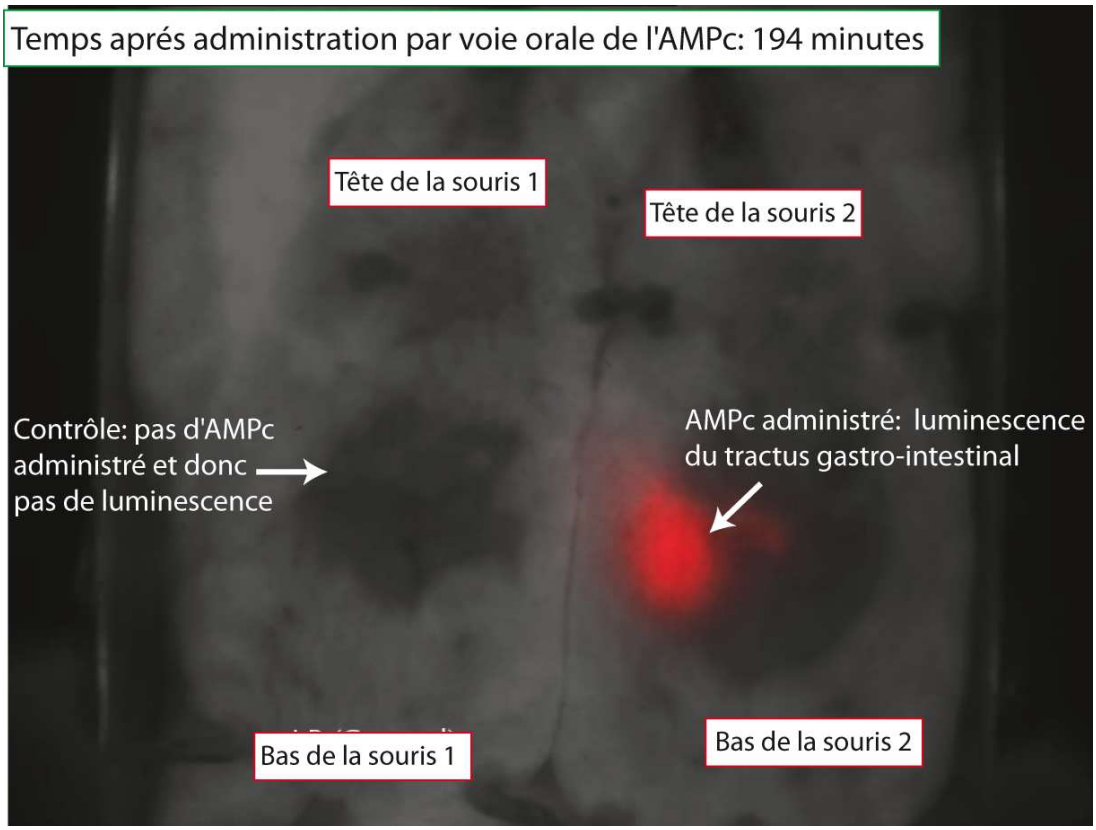

Figure VI.2 – Induction de l'operon *luxCDABE* directement dans le tractus gastro-intestinal d'une souris grâce à l'AMPc. On a administré à deux souris (placées côte à côte sur l'image) des milliards de bactéries $\Delta cyaA$ contenant la fusion transcriptionnelle p-sdh-lux très sensible à l'AMPc extracellulaire. Puis nous avons administré oralement à la souris de droite une forte dose d'AMPc alors que la souris de gauche n'a eu qu'une solution sans AMPc en guise de contrôle. Après quelques dizaines de minutes, on peut observer, de manière transcutanée grâce à une camera sensible, la luminescence émise, depuis le tractus gastro-intestinal, par ces milliards de bactéries dont l'operon *luxCDABE* a été transcrit grâce à la présence d'AMPc.

Dans ces expériences de communication, il perdure une sorte de regret « artistique » : malgré des dizaines de tentatives, nous n'avons jamais pu filmer le processus de communication par l'AMPc, sous le microscope, à l'échelle de la cellule. Nous attendons donc que quelqu'un relève ce défi et nous envoie les vidéos.

Il reste maintenant à décrire les perspectives liées à l'utilisation de l'activité luciferase pour inférer de nombreux paramètres *in vivo* et en temps réel sur le fonctionnement de la cellule. Il s'agit, sans doute, des résultats qui auront le plus d'impact, même si paradoxalement ce sont aussi ceux qui ont été les plus faciles à obtenir. La combinaison de la mesure de l'activité luciferase, des banques de mutants et du savoir actuel sur le métabolisme pourrait aider à générer des données quantitatives plus précises sur l'état des flux métaboliques dans une condition donnée. L'avantage de cette technique est qu'elle ne nécessite pas d'étapes d'échantillonnage et de « quenching » comme c'est le cas pour les mesures de metabolome effectuées *a posteriori* de l'éclatement des cellules.

La capacité à maintenir l'activité luciferase apporte de nombreuses informations *in vivo* et en temps réel : citons l'état énergétique global, l'état du regulon Crp, l'état de la machinerie globale, l'état du milieu, mais aussi des informations plus précises sur l'état d'un flux ou d'une enzyme particulière. La facilité, la sensibilité et le faible coût de détection de la lumière rend notre méthode relativement facile à implémenter. Elle pourrait avoir de nombreuses applications en biotechnologie, en biologie des systèmes ou en biologie synthétique. L'utilisation de notre méthode en fermenteur industriel semble possible *a priori* : il n'est même pas nécessaire que toutes les cellules disposent de la luciferase (ce qui risquerait d'avoir un coût énergétique trop élevé) : une petite proportion de cellules, présentes dans le fermenteur et saturées en luciferase, collecteraient les informations nécessaires, informations elles-mêmes récupérées en temps réel par un simple photomultiplicateur.

A l'opposé, et même si cela nécessite sans doute un matériel plus coûteux, il n'est pas impossible que notre méthode soit implémentable à l'échelle de la cellule isolée. En effet, des cameras sensibles, placées sur un microscope, arrivent à détecter la luminescence de cellule unique (Mihalcescu *et al.*, 2004). Nous pourrions alors suivre, en temps réel, l'état du métabolisme central de cellules isolées ainsi que tous les autres paramètres cités précédemment.

Enfin nous terminerons par vanter les mérites de la fusion transcriptionnelle p-acs-lux rapportant l'activité du promoteur *acs* grâce à la luciferase. Il s'agit d'un bio-senseur robuste et facile à utiliser. Placé dans un milieu minimum avec du glucose et de l'acétate, il rapporte l'épuisement du glucose (induction de la luciferase) et l'épuisement de l'acétate (chute brutale de la luminescence). Placé dans un milieu minimum avec du glucose uniquement, il rapporte la concentration d'acétate sécrétée dans le milieu extérieur c'est-à-dire le débordement du mé-

tabolisme (overflow metabolism). L'accumulation de l'acétate dans le milieu de culture est un problème important dans la fermentation industrielle car cet acide organique inhibe la croissance cellulaire et donc la production des protéines recombinantes. Différentes approches sont actuellement développées pour tenter de réduire l'accumulation d'acétate en modifiant les flux du métabolisme central (Gosset, 2005). Notre bio-senseur pourrait potentiellement aider à mesurer, en temps réel, l'acétate secrété voire même à cribler des banques de mutants à la recherche de candidats intéressants secrétant peu d'acétate.

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L'adaptation d'une bactérie aux changements de son environnement est contrôlée par un réseau de régulation large et complexe, faisant intervenir de nombreux acteurs et modules différents. Dans ce travail, nous avons étudiés un module de régulation spécifique, contrôlant l'adaptation de la bactérie *Escherichia coli* à un changement de sources de carbone. Dans un milieu contenant du glucose et de l'acétate, la croissance est divisée en deux phases : les bactéries utilisent préférentiellement le glucose et commencent à métaboliser l'acétate qu'après l'épuisement du glucose. En effet, la présence du glucose réprime la transcription d'un gène nécessaire à la croissance sur acétate, le gène *acs* (codant pour l'acétyl-CoA synthétase). Le mécanisme régulateur fait intervenir le facteur de transcription Crp-AMPc et le système de transfert de phosphate (PTS), qui permet l'import du glucose.
Plusieurs modèles décrivent en détail la cascade de réactions moléculaires à l'origine de cette « répression catabolique ». Cependant, certaines de nos observations expérimentales ne sont pas correctement prédites par les modèles actuels. Ces modèles doivent être révisés ou complétés. L'outil majeur que nous employons pour les expériences est la fusion transcriptionnelle : une région promotrice fusionnée en amont d'un gène rapporteur (GFP, luciferase). Avec ces constructions, nous mesurons la dynamique de l'expression génique dans différentes souches (mutants) et différentes conditions environnementales. Les observations à l'échelle de la population sont corroborées par des mesures similaires à l'échelle de la cellule unique. Nous utilisons cette même technologie pour construire de petits systèmes synthétiques qui sondent davantage le phénomène de répression catabolique. Nous avons ainsi créé un interrupteur génétique dont le fonctionnement est contrôlé par le flux glycolytique et nous avons construit un petit système de communication intercellulaire basé sur la molécule AMPc. Enfin, nous proposons une manière originale de mesurer l'état métabolique des cellules en utilisant la dépendance énergétique de la luciferase.

Mots clés : Réseaux de régulation, biologie synthétique, cinétique d'expression génique, répression catabolique, gènes rapporteurs

The adaptation of bacteria to changes in their environment is controlled by a large and complex regulatory network involving many different actors and modules. In this work, we have studied a specific module controlling the adaptation of *Escherichia coli* to a change in carbon sources. In a medium containing glucose and acetate, growth is divided into two phases : the bacteria preferentially use glucose and start to metabolize acetate only after glucose exhaustion. Indeed, the presence of glucose represses the transcription of a gene needed for growth on acetate : the *acs* gene (coding for acetyl-CoA synthetase). The regulatory mechanism involves the Crp-cAMP regulator and the phosphate transfer system (PTS), which is responsible for glucose import.
Several models describe the cascade of molecular reactions responsible for this « catabolite repression ». However, our work shows that many of our experimental observations are incorrectly predicted by current models. These models have to be amended. We use transcriptional fusion, i.e., the fusion of a promoter region upstream of a reporter gene (GFP, luciferase), to measure the dynamics of gene expression in different genetic backgrounds and environmental conditions. Observations at the population level are corroborated by similar measurements at the single cell level. We use this same technology to construct small synthetic systems that probe further aspects of the phenomenon of catabolite repression. We have thus created a genetic toggle switch controlled by the glycolytic flux and we have built an inter-cellular communication system mediated by cAMP. Finally, we propose a novel way to measure the metabolic state of cells by using the energy dependence of the luciferase enzyme.

Keywords : Regulatory network, synthetic biology, kinetics of gene expression, catabolite repression, reporter genes