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May 12, 2008

Martine Crasnier-Mednansky
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Dear Dr. Crasnier-Mednansky,

Thank you for contacting PNAS regarding your concerns with the paper by Hiroji Aiba and colleagues titled, "cAMP receptor protein–cAMP plays a crucial role in glucose–lactose diauxie by activating the major glucose transporter gene in Escherichia coli." We shared your concerns with an expert in the field who commented:

> My impression is that the work in question is well regarded and that the arguments made do not hold up.
>
> The writer says that the major problem is the conclusion of this paper that diauxie is not due to reduction in CRP-cAMP levels and is due to inducer exclusion (note that the writer has this wrong and says inducer inclusion).
>
> In fact this conclusion was reached in the predecessor to the PNAS article, published the previous year: Genes to Cells (1996)1: 293 – 301.
>
> The Genes to Cells article measured the levels of cAMP and CRP in glucose media, in lactose media and throughout diauxie conditions; ie during the glucose phase (prelag) and during the lactose phase (postlag) and showed that they were essentially the same, strongly suggesting that cAMP levels are not the reason that lactose is not used when glucose is present. Very importantly, even when cAMP is added to cells given a mixture of glucose and lactose, no β-galactosidase is made when glucose is present. Thus low cAMP cannot explain the *lac* operon is repressed when glucose is present. In this same manuscript, the authors show that eliminating the *lacI* gene (Lac repressor) allows simultaneous use of glucose and lactose, suggesting that somehow the effect is due to inducer exclusion.

Parenthetically, I note that in the Genes to Cells article, Aiba shows that cAMP rises transiently during the lag phase between growth on glucose and growth on lactose, and that addition of cAMP eliminates this lag, most likely by increasing the rate at which β-galactosidase accumulates once glucose is depleted. This is the major point that the writer makes and both he and Aiba are in agreement about these facts. However, the critical point to be explained is hierarchy of sugar utilization and this article convincingly demonstrates that the hierarchy does not rely on cAMP/CRP levels.

The PNAS article continues where the previous article left off and convincingly provides an elegant molecular mechanism for why inducer is excluded. In brief, cAMP-CRP plays an indirect role by activating expression of *ptsG*, the inner membrane component of the best glucose transporter; the cytoplasmic component is IIA^{glc}, (encoded by *crr)*. IIA^{glc} is normally present as the phosphorylated form, but when glucose is being transported the phosphate is transferred to glucose and IIA^{glc} then inhibits *lac* permease. All of the evidence presented is consistent with this. Moreover, and as noted by Aiba, contrary to the writer's claim, elimination of *ptsG* does not eliminate glucose uptake, because there are other lower affinity transporters that play this role. Thus, it is not glucose transport per se but glucose transport via the *ptsG-crr* system that provokes inducer exclusion by creating IIA^{glc}, which is able to inhibit lac permease.

Later work by three groups showed that when glucose is absent, Mlc represses expression of PtsG and IIA^{glc}, facilitating lactose utilization (Aiba, Boos, and Seok).

After considering these comments, we have decided it is not necessary to request a formal response from the authors. We hope that the comments above will address some of your concerns with the article.

Sincerely,

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Editor-in-Chief

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May 15, 2008

Dear Editor-in-Chief,

This letter is my response to your letter dated May 12, 2008 concerning the paper by Hiroji Aiba and colleagues titled, "cAMP receptor protein-cAMP plays a crucial role in glucose-lactose diauxie by activating the major glucose transporter gene in *Escherichia coli*". I would like to thank you for the attention you gave to my concerns, in particular the fact that other researchers are presently basing their work on the publication aforementioned. Herein you will find answers to your expert's response.

I apologize for misspelling 'inducer exclusion'. Any specialist in the field would have known that I meant 'inducer exclusion' and not 'inducer inclusion' (a term which actually does not exist). Amazingly typing 'inducer inclusion' in Google Scholar led me to one article with the same spelling mistake (J Biol Chem, Vol. 274, Issue 10, 6091-6096, March 5, 1999)! By focusing on this triviality the expert unfairly raises suspicion as far as my expertise in the field. This demonstrates partiality.

As explained in great detail below, there are numerous flaws in the Genes Cells article. However, due to your expert's comments I am now compelled to illuminate those flaws. Such action will undoubtedly bring even more embarrassment to the authors. I deliberately chose to ignore the Genes Cells article when I relayed to PNAS the information Ms. Piotrowski had proposed to send the authors. Now I am forced to address issues such as, among other, inappropriate experimental procedures. I believe if the points I raised in my previous letter had not been avoided, I would not have need now to raise the issue to a broader and thus more embarrassing scale.

The most striking inadequacy in the Genes Cells article relates to the interpretation of the data presented in Figure 5. The authors use a Δ*cya crp** strain to analyze diauxie. In a previous 1995 article [Molecular Microbiology] titled, "Glucose lowers CRP* levels resulting in repression of the *lac* operon in cells lacking cAMP", Aiba and colleagues demonstrated that the expression of β-galactosidase is reduced by glucose in cells carrying the *crp** mutations. They proposed that the auto regulatory circuit of the *crp* gene is involved in the down-regulation of CRP* expression by glucose. Conveniently this proposal is not discussed in the Genes Cells article. If however CRP* expression is regulated by glucose in a cAMP-independent manner, diauxic growth observed in Figure 5A does not solely result from inducer exclusion, but also from the effect described in the Molecular Microbiology article. Therefore the authors cannot conclude that inducer exclusion is solely responsible for the diauxie observed in the Δ*cya crp** strain.

Figure 3B of the Genes Cells article reports total and intracellular levels of cAMP during diauxic growth. While the total cAMP level is increased significantly in the lactose phase as compared to the glucose phase, the intracellular cAMP level follows yet another pattern and decreases sharply in the lactose phase. Unfortunately and as clearly stated in a 2007 JB article, measurements of intracellular cAMP concentrations in growing cells is still not achieved in a reliable way. This is due to the fact that excretion of cAMP is extremely active in *Escherichia coli*. It was demonstrated by W. Epstein *et al.* [PNAS] that carbon sources control the intracellular levels of cAMP by regulating its synthesis, and not variations in the efflux rate of cAMP; a result that was later confirmed by studying cAMP transport with membrane vesicles [JB]. Therefore, besides the fact that measurement of intracellular cAMP is unreliable, the abrupt decrease of intracellular cAMP during the lactose phase is highly questionable. Indeed the rate of cAMP excretion is a linear function of its rate of synthesis as previously demonstrated by Epstein *et al.* [PNAS].

It is important that uracil be present in the minimum medium used for growth of strain W3110 (the only wild type strain used by Hiroji Aiba and colleagues) to avoid pyrimidine starvation effects due to a suboptimal content of orotate phosphoribosyltransferase [JB]. Actually it is interesting to note that researchers working with *E. coli* strain W3110 originally thought that uracil was, in general, stimulating the growth rate of *E. coli*. Considering that pyrimidine starvation affects *E. coli* growth, uracil should have been added to the growth medium of strain W3110, especially when studying diauxie which is a growth phenomenon. Furthermore, considering that (1) ppGpp plays a role in the glucose/lactose diauxie [PNAS], and (2) an unusual correlation has been observed between ppGpp pool size and rate of ribosome synthesis during partial pyrimidine starvation of *E. coli* [JB], it seems fundamental to study diauxie with cells that do not starve for pyrimidine.

The experimental data by Ullmann and Monod shows that addition of cAMP eliminates the diauxic lag, and results in a biphasic growth curve (without lag) [Cyclic AMP as an antagonist of catabolite repression in *Escherichia coli*. 1968. FEBS Letters 2:57-60]. Figure 4 of the Genes Cells article does not produce a biphasic curve but a straight linear growth. Again this seems to indicate a problem possibly related to growth conditions used by the authors, and is in contradiction with the established fact that glucose is used before lactose in the presence of cAMP due to inducer exclusion. In these conditions a biphasic curve should have been observed by Hiroji Aiba and colleagues.

Figure 2A of the Genes Cells paper seems irrelevant because it is a well-known fact that, in the absence of induction of the lactose operon, no synthesis of β-galactosidase is observed whatever the level of cAMP. What is relevant is the elegant demonstration by Ullmann and Monod that synthesis of β-galactosidase is dependent on the level of cAMP upon induction with IPTG [Cyclic AMP as an antagonist of catabolite repression in *Escherichia coli*. 1968. FEBS Letters 2:57-60]. In addition, the statement by Hiroji Aiba and colleagues that "the cAMP level in lactose-grown cells was essentially the same as that in glucose-grown cells" is incorrect. The level of cAMP in lactose-grown cells is indeed low as compared to glycerol but higher than the one obtained with glucose-grown cells.

Finally I do not ignore other routes are used for glucose transport in the absence of the glucose permease encoded by *ptsG*. Your expert stated: "... contrary to the writer's claim [myself] elimination of *ptsG* does not eliminate glucose uptake, because there are other lower affinity transporters that play this role". I was extremely careful while wording this part of my original letter to Ms. Piotrowski. What I meant was that the other routes used by glucose do not trigger the glucose effects observed when glucose is transported via the permease encoded by *ptsG*. Therefore I maintain the contention by Hirogi

Aiba and colleagues that “the CRP-cAMP plays a crucial role in diauxie only by activating the glucose transporter gene” (the title of the pNAS article) is absurd.

I implore you to give further attention to this issue, and forward my comments to Hiroji Aiba and colleagues. In a recent 2008 review in Current Opinion in Microbiology titled, “the mechanisms of carbon catabolite repression in bacteria”, it is stated “… repression of catabolic genes was shown to be mainly mediated via inducer exclusion [Ref to Genes Cells]. This aspect is still often wrongly presented in textbooks”. Considering this recent review, I believe the present situation deserves your attention as the implication that textbooks should be modified will definitely jeopardize an already slow progression in the related field. Finally submission of my own papers has greatly suffered from the flawed demonstration by Hiroji Aiba and colleagues that cAMP does not play a role in the exemplary glucose-lactose diauxie.

Yours sincerely,

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May 28, 2008

Martine Crasnier-Mednansky
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Dear Dr. Crasnier-Mednansky,

Thank you for contacting PNAS regarding your concerns with the paper by Hiroji Aiba and colleagues titled, "cAMP receptor protein–cAMP plays a crucial role in glucose–lactose diauxie by activating the major glucose transporter gene in Escherichia coli." We shared your May 15 letter with another member who has provided a preliminary comment. The member noted:

> It is difficult for me to understand why the reader is raising the issues so long after the Aiba paper was published. At first glance, Professor Schekman's response to the reader seems very clear, reasoned, and straightforward. It will take me a while to catch up in depth with the issues raised by the reader. My first thought is that the reader should write a review of the area and try to point out the controversies and the problems the reader perceives with Aiba's previous work. Certainly I don't think PNAS should go back to Aiba over the paper so long after it was published.

We anticipate a more thorough response in early June after the member returns from a trip abroad.

Sincerely,

Randy Schekman
Editor-in-Chief

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June 16, 2008

Martine Crasnier-Mednansky
Chief Science Officer
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Dear Dr. Crasnier-Mednansky,

The PNAS Editorial Board has now solicited the opinion of four different experts on the topic. As you will see in the comments provided below, all agree that the work in question is not sufficiently flawed to justify calling for the withdrawal of the paper by Kimata et al. Instead, the consensus is that interpretations may have changed in the past 11 years but the data stand and should be considered in the context of more recent work.

We appreciate you bringing this matter to our attention and hope you find the comments of the reviewers helpful.

Sincerely,

Randy Schekman

Randy Schekman
Editor-in-Chief

Cc: Dr. David Mednansky

Expert #1 (comments previously sent to Dr. Crasnier-Mednansky on May 12)

My impression is that the work in question is well regarded and that the arguments made do not hold up.

The writer says that the major problem is the conclusion of this paper that diauxie is not due to reduction in CRP-cAMP levels and is due to inducer exclusion (note that the writer has this wrong and says inducer inclusion).

In fact this conclusion was reached in the predecessor to the PNAS article, published the previous year: Genes to Cells (1996)1: 293 – 301.

The Genes to Cells article measured the levels of cAMP and CRP in glucose media, in lactose media and throughout diauxie conditions; ie during the glucose phase (prelag) and during the lactose phase (postlag) and showed that they were essentially the same, strongly suggesting that cAMP levels are not the reason that lactose is not used when glucose is present. Very importantly, even when cAMP is added to cells given a mixture of glucose and lactose, no β-galactosidase is made when glucose is present. Thus low cAMP cannot explain the *lac* operon is repressed when glucose is present. In this same manuscript, the authors show that eliminating the *lacI* gene (Lac repressor) allows simultaneous use of glucose and lactose, suggesting that somehow the effect is due to inducer exclusion.

Parenthetically, I note that in the Genes to Cells article, Aiba shows that cAMP rises transiently during the lag phase between growth on glucose and growth on lactose, and that addition of cAMP eliminates this lag, most likely by increasing the rate at which β-galactosidase accumulates once glucose is depleted. This is the major point that the writer makes and both he and Aiba are in agreement about these facts. However, the critical point to be explained is hierarchy of sugar utilization and this article convincingly demonstrates that the hierarchy does not rely on cAMP/CRP levels.

The PNAS article continues where the previous article left off and convincingly provides an elegant molecular mechanism for why inducer is excluded. In brief, cAMP-CRP plays an indirect role by activating expression of *ptsG*, the inner membrane component of the best glucose transporter; the cytoplasmic component is IIA^{glc}, (encoded by *crr)*. IIA^{glc} is normally present as the phosphorylated form, but when glucose is being transported the phosphate is transferred to glucose and IIA^{glc} then inhibits *lac* permease. All of the evidence presented is consistent with this. Moreover, and as noted by Aiba, contrary to the writer's claim, elimination of *ptsG* does not eliminate glucose uptake, because there are other lower affinity transporters that play this role. Thus, it is not glucose transport per se but glucose transport via the *ptsG-crr* system that provokes inducer exclusion by creating IIA^{glc}, which is able to inhibit lac permease.

Later work by three groups showed that when glucose is absent, Mlc represses expression of PtsG and IIA^{glc}, facilitating lactose utilization (Aiba, Boos, and Seok).

Expert #2

First of all, I think that a retort against a published paper would be better made by presenting data supporting the refutation.

If you ask my own opinion, however, I would say that Aiba's conclusion is somewhat overdrawn. In one hand, I agree with the Editor-in-chief of PNAS that Aiba's work in question is well regarded considering data in PNAS and its precedent paper published in Genes to Cells, although I am not sure how exactly the intracellular cAMP level was measured in media containing glucose, lactose or both in the Genes to Cells paper. It is generally accepted that the cAMP level is usually higher in lactose medium than in glucose medium.

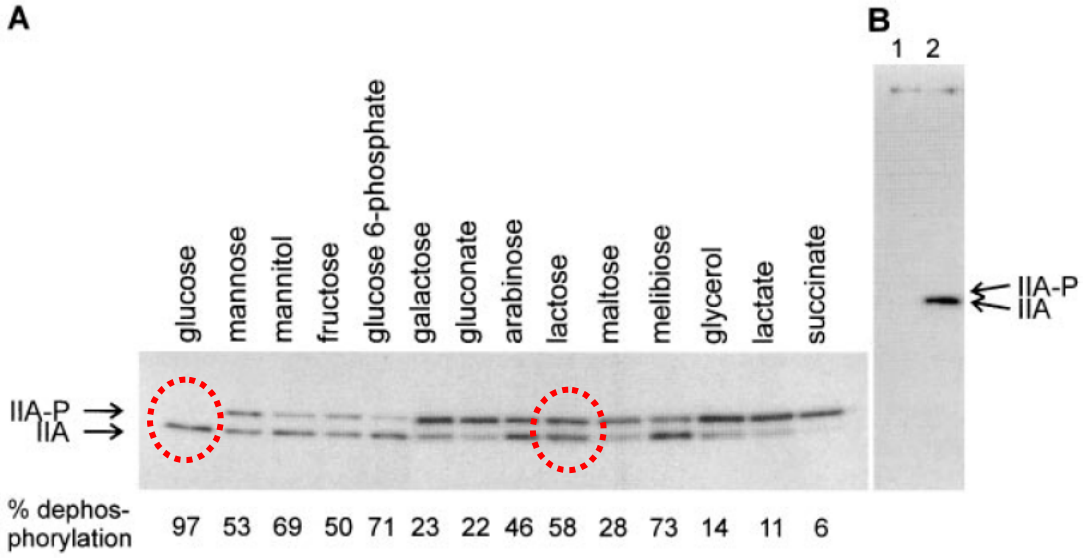
On the other hand, I agree with the reader refuting the Aiba's paper that the problem with Aiba's work is the use of a mutant insensitive to cAMP levels.

It is well established that cAMP induces expression of the *lac* operon including the *lacZ* gene. It was shown that, in a medium containing both IPTG and glucose, expression of beta-galactosidase was increased by the addition of cAMP in milestone papers (Pastan and Perlman, 1968, PNAS 61: 1336; Ullmann and Mond, 1968 FEBS Letter 2: 57).

Expression of PTS is known to be positively regulated by CRP-cAMP, which decreases in the presence of glucose, and negatively regulated by Mlc, which is inactivated by PtsG dephosphorylated in the presence of glucose. Therefore it can be expected that the addition of cAMP to the culture medium containing both IPTG and glucose will further increase PTS expression and the total amount of dephospho-Crr. If CRP-cAMP plays a crucial role in diauxie only by activating the *pts* operon and *ptsG* gene, then addition of cAMP should further stimulate inducer exclusion and thus further decrease expression of beta-galactosidase. However, the opposite happened in classical experiments.

Now we know that the phosphorylated form of Crr activates adenylyl cyclase (AC) whereas only the dephospho-fom of Crr blocks entry of inducer.

Let's say that the ratio of phospho- to dephospho-Crr (P/D) in glucose medium is 5% and that in lactose medium is 50% as in Mol Microbiol 30:487 where Aiba is a co-author (Figure below). Then the ratio of P-Crr in glucose to that in lactose medium is about 1:10 while the ratio of D-Crr is only 2:1. This means that the concentration of dephospho-Crr decreases only two fold when medium is shifted from glucose to lactose, while that of phospho-Crr increases about 10 fold. In other words, AC activity can increase up to 10 times, while inducer exclusion can decrease at most 2 times on glucose-lactose shift. There is still plenty of dephospho-Crr which can block the entry of the inducer in a lactose medium. Therefore, it is assumed that inducer exclusion alone is not enough to fully explain glucose-lactose diauxie.



Again, I think that Aiba's team made reasonable interpretation about their data and a retort against a published paper would be better made by presenting data supporting the refutation. To draw a convincing conclusion, more experiments would be necessary. I doubt whether IIA will mainly exist in the phosphorylated form in a delta-crp strain growing in a glucose medium and a delta-mlc mutant, which makes more PtsG, will be resistant to catabolite repression.

Expert #3

I believe that the request by Dr. Crasnier-Mednansky to contact Dr. Aiba requesting that he submit a "correction" to his 1997 PNAS paper is not justified. I will not reiterate the comments of the expert in the field and do agree that the 1997 paper is a solid, although not field-encompassing, study in an area that is still not totally defined. As pointed out by the expert, there were subsequent findings implicating Mlc in the expression of proteins relevant to the inducer exclusion mechanism.

Every few years, a group of investigators prominent in the field of bacterial phosphotransferase systems has undertaken the herculean task of assessing the current status of the connection between the phosphotransferase systems and the regulation of carbohydrate metabolism. The most recent review in this series (Microbiol. Mol. Biol. Rev. 2006 Dec. 70 (4) 939-1031) points up the complexity in this area and provides a comprehensive assessment of the current understanding of the catabolite repression and inducer exclusion phenomena. It is my impression that interested workers in the field consult and refer to this work as the standard. A request to Dr. Aiba to refine the interpretation of his results from 1997 in the context of today's knowledge appears to me to be a non-productive approach to advance the field and should therefore not be granted.

Expert #4

I have looked into the matter of the Aiba paper (Kimata K, Takahashi H, Inada T, Postma P, Aiba H. cAMP receptor protein-cAMP plays a crucial role in glucose-lactose diauxie by activating the major glucose transporter gene in Escherichia coli. Proc Natl Acad Sci U S A. 1997 Nov 25;94(24):12914-9) more carefully. Given that it is 11 years since this article was published in the PNAS, my recommendation remains that an expert review is the best means to scientific progress in the field of the glucose-lactose diauxie in *E. coli* and the role(s) of c-AMP and its binding protein in this diauxie. A review could clarify the issues in dispute, the data in dispute, the interpretations in dispute, and perhaps include suggestions for additional experiments that might resolve the issues. It would be an excellent and appropriate means of advancing research in the area.

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June 19, 2008

Dear Editor-in Chief,

I am in receipt of your answer in relation to the PNAS article by Hirogi Aiba and Colleagues (your letter dated June 16, 2008). I was extremely surprised to learn that you concluded the work in question is not "sufficiently flawed". A statement like this is not expected from an editor of a journal that has been held in high regard by the scientific community. Your statement begs questioning which criteria you use to determine the unaccepted flaw level of a paper. In fact it begs questioning why any known flaw is acceptable.

Your statement that "interpretation may have changed in the past 11 years" is incorrect. There has been a limited amount of papers dealing with diauxie within the last 11 years. Moreover to my knowledge there are no data that do not support the "dogmatic" interpretation of diauxie, not even Hirogi Aiba and colleagues' data. I have stated before that the flaws were in the interpretation of the data, not merely the data themselves. Furthermore time period interpretation was incorrect considering the well-established research prior to publication. Therefore your conclusion that "the data stand and should be considered in the context of more recent work" demonstrate a lack of focus on the issue at hand.

The discovery by Jacqueline Plumbridge (Molecular Microbiology, 1998, 29: 1053-1063) that expression of the gene encoding the PTS-glucose permease is repressed by Mlc, and induced by growth on glucose, is relevant to diauxie. However it cannot cause a re-interpretation of diauxie in mechanistic terms, but it leads to a more accurate analysis of diauxie in kinetic terms. Indeed diauxie is a quantitative phenomenon, the glucose/lactose ratio determining the extent of diauxie. As such the transcriptional regulation of *ptsG* (encoding the PTS-glucose permease) is to be construed, in diauxie, as controlling the rate of glucose transport, which is linked to both inducer exclusion and regulation of adenylate cyclase.

Finally, there is an urgency to deal with this issue. The expert has stated that "the Aiba's work in question is well regarded" (Expert #2) therefore urgency and relevance is demonstrated by your own expert.

Please find attached my answer to the experts' comments.

I hope you can understand my growing frustration in dealing with this issue which, although I consider it my duty in the interest of science, is now taking too much time. Therefore it should be dealt with swiftly in any manner necessary to facilitate retraction.

Sincerely yours,

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Expert #1:

I have already answered Expert #1 (my letter dated May 15 , 2008). Expert #1 response is not directed at my scientific reasoning for retraction.

Expert #2:

Data supporting my call for retraction had been published 'extensively' prior to Dr. Aiba's papers. There is absolutely no need to present new data because data regarding my position on this issue were well established prior to Dr. Aiba's work. Subsequently Dr. Aiba had ample opportunity to be knowledgeable about these data. One of my papers on diauxie showing new data, which were in sharp contradiction with Dr. Aiba's interpretation of diauxie, was rejected three times for publication; no need to mention why. My first submission was at the time the Aiba paper was published.

I am glad the expert acknowledged there is validity to the points I have raised. The expert confirms the fact that "the problem with Aiba's work is a use of a mutant insensitive to cAMP levels". This is clearly a 'major' flaw in Aiba's work. The expert also confirmed that "if CRP-cAMP plays a crucial role in diauxie only by activating the *pts* operon and *ptsG* gene, then addition of cAMP should further stimulate inducer exclusion and thus further decrease expression of beta-galactosidase (thereby enhancing the diauxic lag, my words). However, the opposite happened in classical experiments (where cAMP eliminates the diauxic lag, my words)". This clearly corroborates my contention that the title of the PNAS article is absurd, and most importantly totally misleading.

I am relieved that the expert pointed out the discrepancies among Dr. Aiba's papers (the expert's paragraph 7 and figure attached). This perspective is in accordance which what I had outlined in my previous correspondence for a parallel issue. The expert states that "inducer exclusion alone is not enough to fully explain glucose-lactose diauxie". The 1998 results in Mol Microbiol 30:487 actually support the view that both inducer exclusion and cAMP levels are responsible for diauxie, but in any case offer new insights for re-interpreting the phenomenon of diauxie.

In this context it is illogical as to why the expert, considering his argumentation, concludes that "Aiba's team made reasonable interpretation about their data". How can Aiba's flawed interpretation be contrived to be reasonable by the expert since the expert has demonstrated numerous flaws in Aiba's interpretation?

Expert #3:

First, it is interesting that the expert refers to the 2006 MMBR review as the standard. As far as diauxie is concerned there is a gross error in this review. *E. coli* does not exhibit diauxie in the presence of fructose and a less favorable sugar (as stated in the introduction and discussed in the text of the review). I have been in communication with the corresponding author of the review who in turn has promised a retraction. However any expert in the field should have been able to see this error on their own.

Second, Dr. Aiba ignored previously published data, including his own, which are in conflict with his interpretation without explanations. Therefore, it is not a refinement of his interpretation in the

context of today's knowledge that is needed, but a full retraction of his interpretation in the context of past published knowledge. Because of the flawed interpretations by Dr. Aiba in both Genes and Cells and PNAS papers, the cAMP-related community has inevitably suffered. For example, in the MMBR review there is a respectable attempt by the authors to reconcile the discrepancy between Dr. Aiba's data and previously published data. What the authors of the MMBR review failed to realize however is the fact that Aiba and colleagues did not use the proper strains to address the issue at hand (as already explained in my previous correspondence). Furthermore there is no need to drag out this issue any further since my scientific statements have not been proven unsound. Moreover the expert has not given adequate scientific justification to prevent retraction. This issue is best resolved by asking Dr. Aiba to address the issues I have raised.

Expert #4:

Eleven year passage of time alone does not right a wrong. Especially since all evidence I have provided substantiate the misleading direction contemporary scientists have and are being sent in. Furthermore what assurance is there that a new review by a current expert would be accepted? Indeed, I have recently submitted a review on diauxie which was rejected because of Dr. Aiba's flawed demonstration that cAMP does not play a role in the exemplary glucose-lactose diauxie. My paper was rejected for emphasizing the cAMP effect in diauxie.