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Page limits do not include references, tables, charts, figures and photographs. However, legends to the latter should be succinct. Detailed descriptions of methods and discussion of results should be included in the body of the proposal, NOT in the legends. Questionnaires and consent forms may be attached as appendices where applicable.

Regulation of Competence in *Haemophilus influenzae*

Our goal is to understand how *Haemophilus influenzae* regulates its ability to take up and recombine DNA from its environment. This is important for two reasons: (i) The immediate benefits of DNA uptake are nutritional, so understanding competence should clarify how *H. influenzae* and other mucosal pathogens exploit their specific environments; (ii) Transformation is a form of genetic exchange, and understanding its control will illuminate both the role of genetic exchange in bacterial evolution and the function of sex in eukaryotes (see Redfield 2001 (provided as #1) and the collection of articles in¹).

H. influenzae is a small gram-negative bacterium frequently commensal in the human upper respiratory tract, and is a common cause of serious infections. Serotype b strains are the major cause of meningitis in infants and small children, with a 6% mortality rate and residual damage to hearing or intellect in about 50% of cases². A conjugated anti-type b vaccine effective in infants is now available, but there is no vaccine for other serotypes, which are important causes of childhood ear infections and of respiratory disease in infants, the elderly and people with AIDS³. Competence and transformation have medical relevance at several levels, as antibiotic resistance genes, virulence determinants and capsular serotype genes are spread by transformation^{4,5,6,7}. Once understood, DNA uptake pathways could also provide routes for antibiotic delivery. Our working hypothesis is that competence is part of a suite of adaptations to a mucosal environment, allowing cells to use the abundant host DNA in respiratory tract mucus as a source of nucleotides. Competence is regulated by metabolism, so dissecting the regulatory signals will clarify the control of fundamental processes in this pathogen.

Background:

The ability to actively take up DNA molecules from the environment is widely distributed among both gram-positive and gram-negative bacteria, but its significance is not understood. The regulation and mechanism have been well-characterized in only a few groups, primarily the gram-negative *H. influenzae* (and to a lesser degree *Neisseria*, *Acinetobacter*, *Helicobacter* and *Pseudomonas* species), and the gram-positive *Bacillus subtilis* and *Streptococcus pneumoniae*^{8,9,10}. Natural competence should not be confused with the artificial cell-permeabilization procedures used to introduce plasmids into many types of cells. The discussion below is restricted to *H. influenzae*; competence in other bacteria is reviewed in references 8, 9, and 10.

How does *H. influenzae* take up DNA?

Figure 1 summarizes the steps involved in competence and our knowledge of the genes responsible. (A detailed table of all known and candidate competence genes is available at www.zoology.ubc.ca/~redfield/CIHR, along with some recent papers and other supporting materials.) Cells develop competence in response to changes in growth conditions, discussed in detail below. Once competent, cells bind up to 300kb of double-stranded DNA, preferentially binding fragments carrying the uptake signal sequence AAGTGCGGT (the 'core USS'), which is highly over-represented in the *H. influenzae* genome^{11,12}. (In a separate research project funded by NIH we are investigating the mechanism of DNA uptake and the role of this uptake signal system

in both *Haemophilus* and *Neisseria* (*proposal is on web page*.) DNA uptake is a two-stage process; fragments are first transferred intact into what is probably the periplasmic space, where the DNA is inaccessible to both extracellular DNases and cytoplasmic restriction enzymes¹³. Next, one strand is degraded as DNA is translocated into the cytoplasm and the resulting free nucleotides are rapidly reused for new DNA synthesis. The remaining strand may recombine into a homologous segment of the chromosome (the resident strand is displaced and degraded); otherwise it too is degraded and reused¹⁴. A cell whose genotype is changed by this recombination is said to be transformed. Procedures for measuring competence are described in #2.

Regulatory and mechanistic genes: A key distinction for our studies is between the genes directly responsible for DNA uptake and the regulatory genes that control their expression. This distinction can be difficult to make, as a cell may fail to take up DNA because its uptake machinery is faulty or because it cannot produce or recognize competence-inducing signals. We have mainly used the *comABCDEF* operon (henceforth called *comA-F*) and the *rec-2* gene as examples of competence-regulated mechanistic genes. Insertions in the *comA*, *comC* and *comE* genes prevent DNA binding and uptake; insertions in *comF* and *rec-2* prevent translocation into the cytoplasm^{15,16}. Both promoters are expressed at very low levels in exponentially growing cultures and highly induced in competent cells¹⁷, and we have used knockout mutations and *lacZ* fusions to show that neither plays a role in regulating the other.

When does *H. influenzae* become competent?

In the laboratory, *H. influenzae* cells are usually grown in brain-heart infusion broth with added hemin and NAD (sBHI). Log-phase cells are not competent; DNA binding cannot be detected and transformation frequencies are below the usual detection threshold of 10^{-8} . As cells approach stationary phase about 1% of cells become competent, giving transformation frequencies of about 10^{-4} . Maximum competence is induced by transfer of log-phase cells to a starvation medium called M-IV ("M-four"), which contains amino acids but no carbohydrates, nucleic acids or cofactors¹⁸. Most cells in the culture become competent within 100 minutes of transfer to M-IV. Cells growing in colonies on agar also become competent¹⁹, with transformation frequencies of about 10^{-5} - 10^{-3} . Competence phenotypes of mutant cells range from complete non-competence (TF 10^{-8} in M-IV) to extreme hypercompetence (TF 10^{-3} in exponential growth).

Why does *H. influenzae* become competent?

Researchers have given little thought to the evolutionary forces that shaped natural competence. Here we explicitly consider three possible functions of DNA uptake and transformation and the modes of regulation they predict. The issue is discussed in more detail in #1.

Possible function 1. Genetic recombination: Most biologists have assumed that competence evolved to allow transformation to create recombinant genomes. However, research into the evolution of sex has had great difficulty finding benefits that outweigh the costs²⁰, and our theoretical work extending this analysis to transformation reveals significant new genetic costs^{21,22} (*pdf on web page*). Most hypotheses about the benefits of recombination can only be tested experimentally in bulk cultures and in chemostats, conditions highly prone to cryptic selection and other experimental artifacts²³.

Possible function 2. Recombinational DNA Repair: Competent cells may use some of the DNA they take up as templates for recombinational repair of otherwise irreparable DNA damage²⁴. Such physical lesions are very frequent and lethal if unrepaired, so use of incoming DNA for repair is potentially more beneficial than recombination. However, uptake of homologous DNA does not detectably increase *H. influenzae*'s ability to survive DNA damage²⁵, and competent *H. influenzae* and *B. subtilis* cells are actually less able to repair UV-induced DNA lesions than are normal cells^{26,27}.

Possible function 3. Nucleotide Salvage from DNA: One reliable benefit of DNA uptake is the nucleotides released when the DNA or the strand it has displaced is degraded inside the cell. *H. influenzae* has the genes to synthesize its own nucleotides²⁸, but calculations done for *E. coli* indicate that this will consume about 16% of the cell's total energy budget²⁹. It is thus not surprising that, like other bacteria, *H. influenzae* makes efficient use of DNA and RNA precursors provided in the culture medium. However, although its natural environment is very rich in DNA (300µg/ml)³⁰, it does not secrete nucleases that would hydrolyze the DNA for uptake by standard salvage pathways³¹. This may be because DNA taken directly into the cell is hydrolyzed to dNMPs, bypassing the degradation to bases plus deoxyribose and resynthesis required when dNMPs are generated externally and then imported. Uptake of DNA also avoids losses due to extracellular diffusion of nucleases and nucleotides. Use of DNA for carbon, nitrogen, or energy rather than nucleotides can also be efficient, but only if DNA is in excess of the cell's need for nucleotides.

Competence is tightly regulated by mechanisms that respond to the shifting balance of benefits and costs associated with DNA uptake. Thus each of the above roles for competence makes predictions about regulation. Function 1: Because the costs and benefits of recombination are still controversial, regulatory predictions based on them are highly speculative. The most plausible is that DNA uptake should be induced only "when all else fails", *i.e.* only when multiple global regulators (SOS, the stringent response, catabolite repression, heat shock, Lrp) signal that they are unable to maintain growth or survival. However, in *H. influenzae* competence is known to not be regulated by SOS, stringent response or heat shock. Function 2: The DNA repair hypothesis makes a simple prediction: competence should be inducible by DNA damage. However our experiments in *H. influenzae* and *B. subtilis* have shown that this is not the case, although both species have the components needed for such regulation: inducible competence and damage-inducible SOS responses³². Thus any benefits from DNA repair are probably minor. Function 3: If the primary benefit were acquisition of nucleotides by DNA salvage, regulation should tie competence to both energy resources and the availability of DNA precursors. Under this scheme, nucleotides or bases would be taken up when available, and, when pools became depleted, synthesized *de novo* or salvaged from extracellular DNA depending on availability of energy resources. The observed regulation fits this prediction.

How does *H. influenzae* regulate competence?

We begin here with the simple model presented in our 1995 MRC proposal (**Fig. 2**) and develop the unresolved issues that our experiments will address. **Fig. 3** presents our current model.

Simple model: (**Fig. 2**). Competence is regulated by controlling the transcription of many of the genes needed for DNA uptake. The promoters of these regulated genes are preceded by a conserved DNA sequence element, the CRE site (Competence-Regulated Element, see **Figs. 1 and 5**)^{33,34}. Below we refer to these as "CRE promoters" and "CRE genes". Transcription of CRE genes requires the activator proteins Sxy and CRP, whose activities reflect reduced availability of preferred sugars, signaled by a rise in intracellular cAMP, and possibly of nucleotides.

Complexities of regulation of cAMP: In enteric bacteria the phosphotransferase sugar-uptake system (PTS) controls intracellular cAMP levels, and these determine the activity of CRP. As we had hypothesized, the PTS acts similarly in *H. influenzae* to regulate the production of cAMP; see **#3**^{35,36}. The only PTS-transported sugar is fructose—the usual glucose permease is missing from the *H. influenzae* genome though it is present in the other sequenced members of the Pasteurellaceae, *Pasteurella multocida* and *Actinobacillus actinomycetemcomitans* (henceforth called *A. actino*). The regulatory subtleties of the PTS have not been clarified; excessive cAMP

levels have partially-inhibitory effects which we will not investigate further³⁶. Levels of cAMP are also controlled by the activity of a cytoplasmic cAMP phosphodiesterase encoded by the *icc* gene³⁷. It likely functions to prevent cAMP from accumulating and (masking rapid changes in cAMP production. There is no evidence that the phosphodiesterase is itself regulated and we will not study it further.

Complexities of regulation of *sxy* expression: The Sxy protein (also known as TfoX³⁸) is required for competence and for transcription of *comA-F*, *dprA* and *rec-2*. If Sxy's function is to regulate competence (no other function is known), then its expression or activity must vary in response to as-yet unidentified environmental or intracellular cues. Transcription of *sxy* was originally reported to be regulated by CRP³⁸. However, Laura Bannister, a former PhD student, has carried out a detailed analysis of *sxy* expression using transcriptional and translational fusions to *lacZ*. Expression of *sxy* was not changed by either supplementing medium with cAMP, which stimulates competence, or knocking out *cya* (adenylate cyclase), which eliminates it³⁹. Her analysis showed that expression of *sxy* does depend on culture density, but the main increase in transcription occurs much earlier than the expression of CRE genes. Expression from a Sxy-protein fusion increased again at higher cell densities, suggesting that translational regulation might contribute to induction.

We originally identified the *sxy* gene as the site of a mutation that causes hypercompetence^{19,40}. Since then we have identified additional hypercompetence mutations in *sxy*. All are single-nucleotide substitutions; only *sxy-1* changes the amino acid sequence. Their only commonality is that each would weaken a potential secondary structure in the 5' end of *sxy* mRNA, which we hypothesize to have a regulatory role (**Fig. 4A**). Laura Bannister showed that the original hypercompetence mutation, *sxy-1*, increases transcription and especially translation (**Fig. 4C**), as does elimination of the stem (**Fig. 4B**), and she confirmed the importance of the secondary structure by creating compensatory mutations which reduced expression and dramatically reduced or eliminated competence. The secondary structure may serve a genuinely regulatory function, perhaps by coupling stability or translatability of *sxy* mRNA to the effect of nucleotide pools on transcription, or it may simply set the baseline level of expression.

Many bacteria have *sxy* homologs, but no mutants have been isolated and nothing is known about its function or regulation in other organisms. The *E. coli* homolog is particularly interesting because *E. coli* also has homologs of *H. influenzae*'s *comA-F*, *rec-2* and *comJ* genes, and requires some of these to use DNA as a nutrient⁴¹.

Regulation by Sxy and CRP/cAMP at CRE promoters: J.F. Tomb first pointed out that the promoters of genes known to play roles in competence shared a common sequence element, the CRE^{42,33,34}. Both Sxy and CRP are needed for transcription of those CRE promoters that have been examined using *lacZ* fusions (*comA-F*, *dprA*, *rec-2*), and mutations in most CRE genes are known to prevent competence (*ssb* and *pbpG* have not been examined). The requirement for both CRP and Sxy was originally explained by having CRP induce transcription of *sxy* and Sxy then induce transcription of CRE promoters^{42,33}. However Sxy is unlikely to bind unassisted to CRE promoters as it bears no resemblance to known DNA-binding proteins³⁹. Furthermore, CRE sites appear to be a subclass of CRP binding sites (**Fig. 5**) sufficiently similar that failure of CRP to bind at them would be surprising³⁴ (*pdf on web page*). In combination with the CRP-independence of *sxy* transcription, this suggests the model shown in **Fig. 3**, in which CRP induces transcription at CRE promoters, but only in the presence of Sxy. (We considered whether FNR might bind to the CRE or otherwise affect competence, because FNR is a transcriptional activator in the same family as CRP and senses the availability of oxygen, a factor known to influence competence development. However a FNR knockout did not change competence.)

Regulation by nucleotide pools: If cells do use DNA uptake to obtain significant amounts of nucleotides, nucleotide pools should regulate competence. Paper #4 shows that addition of purine nucleotides reduces competence 200-fold and represses transcription of *comA-F* and *rec-2*⁴³, and suggests that the purine-sensing repressor PurR mediates this repression. A new PhD student, Kenith Chan, has now knocked out the *purR* gene and shown that this relieves repression of *rec-2*. However it does not restore competence or relieve repression of *comA-F*, implying the existence of a PurR-independent component of nucleotide regulation.

Added cAMP overcomes repression of *comA-F* by GMP and, to a lesser extent, by AMP (#4), suggesting that the second component could be reduced cAMP production or interference with CRP. Alternatively, nucleotides could reduce Sxy levels, acting via the secondary structure of *sxy* mRNA. Precedents occur in the coupling of transcription and translation in genes for nucleotide biosynthesis, where translation occurs efficiently only when depleted nucleotide pools limit transcription^{44,45,46,47}.

Regulation by cell wall metabolism: Our screens for mutations that enhance competence produced four isolates with point mutations in the *murE* gene, which encodes a step in peptidoglycan biosynthesis (**Fig. 6**). The analysis described in #5 showed that the mutations do not detectably alter cell wall permeability or stability, but do dramatically increase transcription of *comA-F* and *rec-2*. Involvement of MurE in competence regulation is unexpected, as it was thought to have only a catalytic role. The precise locations of the *murE* mutations do not help explain their consequences—a mutation replacing a highly-conserved leucine with a serine causes the same phenotype as mutations in a poorly-conserved region. We have focused on the *murE*₇₄₉ mutation. In complementation tests it behaves as a loss-of-function mutation with respect to its effect on competence, but catalytic function is apparently unimpaired.

Our experiments to date have failed to support our hypotheses. These start with the assumption that the mutation modestly decreases MurE's catalytic activity, creating a bottleneck that leads to changes in gene expression. The first hypothesis we tested invokes the link between murein recycling and gene regulation diagramed in **Fig. 6**. However knockouts of the recycling genes have only minor effects on competence (#5). Recently we have also knocked out *gcvA*, a LysR-family transcriptional activator that can sense a murein-recycling intermediate and regulate transcription of beta-lactamase genes⁴⁹. *GcvA*'s targets in *E. coli* are absent from *H. influenzae*, opening the possibility that its main role in *H. influenzae* is regulation of competence in response to changes in cell-wall metabolism. However the *gcvA* knockout reduced competence only 5-10-fold. Andrew Cameron, a new PhD student, constructed a *murE*₇₄₉/*gcvA* double mutant; this has the same elevated competence as its *murE*₇₄₉ parent. Adding the MurE substrate diaminopimelate (DAP) to culture medium had no effect on competence, so *murE*₇₄₉'s effects are not due to DAP accumulation. A possible effect of accumulation of the sugar-dipeptide substrate has not been tested. Effects on competence could be indirect; a bottleneck at MurE may increase transcription of the complex *mra* cluster of murein synthesis and cell division genes (**Fig. 6**). Regulation of and by these genes is complex and one or more could be responsible for the effect on competence⁵⁰. CRE sites and other mutations confirm that murein metabolism plays an important role in competence (*pbp1*¹⁵, *pbp2*, *rodA*⁵¹, *pbpG*³³), so this problem continues to have high priority.

Mutations in *trmE*, *rpoBC* and *fis* reduce transcription of competence genes: MiniTn10kan insertions in *trmE* (formerly called *thdF*) and between *rpoB* and *rpoC* were isolated in 1989, but the affected genes were not identified at that time⁵². Mutations at both sites eliminated DNA uptake, but it was not known whether the effects were regulatory or mechanistic. We have now mapped and characterized them. Seven closely linked insertions are all in the open reading frame

HI1002, which belongs to a family of highly conserved bacterial GTPases⁵³. The *E. coli* member, *trmE* (formerly called *thdF*) is involved in tRNA methylation and translational frameshifting⁵⁴. The *H. influenzae* mutations dramatically reduce transcription of *comA-F* and *rec-2*. We mapped another miniTn10kan insertion to the untranslated region between the *rpoB* and *rpoC* genes (HI0515 and HI0514); it also prevents competence-induced expression of *comA-F* and *rec-2*. Insertions at both locations reduce growth, and one explanation for their effects on competence is that they alter the regulation of *sxy*.

Laura Bannister showed that a *fis* knockout reduces competence by several hundred fold³⁹. Transcription of *comA-F* and *rec-2* is reduced but transcription of *sxy* is not. Fis is a DNA-binding protein with modest sequence specificity; it contributes to the activities of many more specific DNA-binding proteins. Its pattern of expression is the inverse of Sxy; it is abundant in early-log cells but decreases as density increases.

Competence in other bacteria:

A number of *H. influenzae* isolates are only poorly competent, and some cannot become competent at all. The problem is not the presence of type-specific capsule or other virulence factors. Competence is also sporadically distributed among other members of the *Pasteurellaceae*^{55,56}, and nontransformable isolates are also common in other 'transformable' bacteria: *Streptococcus*⁵⁷; *B. subtilis*⁵⁸; *N. gonorrhoeae*⁵⁹. The inability of some strains to be transformed poses evolutionary questions as well as experimental problems: Have some strains lost the ability to take up DNA, or is the problem only methodological? If the latter, can the methods be improved? Is loss of transformability caused by phage lysogeny⁶⁰? Are competence genes frequently deleted because in some circumstances DNA uptake is consistently harmful? If so, perhaps this could be exploited therapeutically.

Specific questions to be answered:

- 1. Which competence genes are regulated by PurR?**
- 2. Do nucleotide pools control (i) cAMP levels? (ii) *sxy* expression?**
- 3. Does Sxy (i) control transcription of all CRE genes? (ii) control transcription of any non-CRE genes? (iii) act only with CRP/cAMP?**
- 4. How do Sxy and CRP control transcription from CRE promoters?**
- 5. How does *murE* control transcription from CRE promoters?**
- 6. Why are some *H. influenzae* isolates not competent?**

Experimental approaches:

Standard culture methods and genetic techniques are described in the *H. influenzae* methods chapters provided (#2). Only methods not covered there are described here. Bandshift protocols are based on those used to analyze CRP interactions⁶¹. Dr. Spiegelman's laboratory will provide support with bandshift assays (*letter attached*). Anti-peptide antibodies to Sxy and CRP will be obtained from Alpha Diagnostic International (www.4adi.com). They have recommended suitable peptides based on the predicted hydrophilicity, antigenicity and accessibility profiles of each protein. A sheet documenting their services and charges is attached to the Budget. Dr. Matsuuchi will provide assistance with Western blotting (*letter attached*).

Strategy for microarray analysis:

Source of microarrays: We are extremely fortunate in having access to *H. influenzae* microarrays produced by the Wellcome Trust Multi-collaborative Microbial Pathogen Microarray Facility (See http://www.sghms.ac.uk/depts/medmicro/bugs/bugs_content_frame.htm). As a collaborating partner to the array consortium we have access at nominal cost to arrays containing all 1738 *H.*

influenzae Rd genes as well as some genes from the pathogenic serotype b strain Eagan, and will share our experiences and data with the other members of the consortium (letter attached).

Data generation: RNA isolation and probe labeling protocols are as follows: Total bacterial RNA will be isolated by guanidinium-thiocyanate-phenol-chloroform extraction (TriSol) followed by DNase I digestion, phenol extraction and ethanol precipitation. cDNA will be synthesized from total RNA using SuperScript II reverse transcriptase and a nucleotide mix containing Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia) to prepare probes for array screening. The Wellcome Trust consortium has found that random-hexamer priming of bacterial RNAs is satisfactory. The two cDNA pools to be compared will be mixed, applied to the array, and competitively hybridized⁶². After washing, arrays will be scanned at the microarray facility maintained by the Prostate Cancer Centre at UBC, which will also provide us with local support (*letter attached*). (*The technician will be responsible for probe preparation, hybridizations and scanning.*)

Data analysis: Microarray data will be analyzed with GeneSpring software, a cross-platform package also being used by the Prostate Centre and the Wellcome Trust consortium. Depending on the experiment, data analysis will be done by the technician or by the student or post doc most concerned. Because most of the data will be relevant to more than one question, everyone in the lab will have access to it and will be trained in analytical techniques.

Preliminary microarray experiments: The Budget Module includes a table showing all the planned microarray experiments. The first will compare RNAs from cells in exponential phase ($\sim 5 \times 10^8$ cfu/ml) in sBHI and from competence-induced cultures (50 min in M-IV). We expect the 'learning curve' to consume between five and ten arrays. The next step will be a time course of transcription of competence-related genes, to establish basic expression patterns and identify the best times for sampling cultures in subsequent experiments. For this analysis an exponentially growing culture will be sampled at cell densities of 2×10^8 and 5×10^8 cfu/ml. One half of the culture will then be transferred to M-IV. Additional samples will be taken after 10, 30, 60 and 100 minutes in M-IV, and after 45, 90 and 150 minutes in rich medium, when the competence of each culture will be at its peak. Labeled cDNA from each sample will be competed with a standard labeled cDNA made from a mixture of the previously characterized exponential-phase and competence-induced RNAs. Detailed analysis of the data will reveal the expression kinetics of known competence genes, and identify any previously-unknown genes induced in competent cells⁶³.

Research Questions:

1. Which competence genes are regulated by PurR?

We began with the simple hypothesis that purine nucleotides and nucleosides inhibit competence because some competence genes have operator sites for the PurR repressor⁴³ (#4). Construction of a *purR* knockout strain confirmed that PurR represses *rec-2* transcription. This will be further verified by mutagenizing the postulated PurR site in its promoter, which should restore expression in the presence of nucleotides. Like *rec-2*, *dprA* contains an appropriately positioned PurR box and is needed for translocation of DNA into the cytoplasm in *H. influenzae* and other bacteria^{64,65}; the role of PurR will be tested in the same way.

The program PatSearch (available at <http://bigghost.area.ba.cnr.it/BIG/Patsearch/>) will be used to search for previously unrecognized PurR boxes. Mironov *et al.* only considered genes whose *E. coli* homologs were known to be under PurR regulation and so did not find the PurR boxes in the *rec-2* and *dprA* promoters⁶⁶. PatSearch searches the EMBL databases for matches to user-specified DNA or protein sequences or sequence patterns; it can restrict searches to prokaryotic sequences and accepts a weighted sequence matrix of the form shown in Fig. 5. Each match produces an EMBL

accession number, the name of the organism, and the position and sequence of the pattern match, which can then be located with respect to predicted promoters. PatSearch will also be used to examine the genomes of *P. multocida* and *A. actino* for PurR boxes, and new competence genes with apparent PurR boxes in all three species will be investigated using gene-specific methods.

Microarrays will be used to compare expression of *purR*⁺ and *purR*⁻ strains in M-IV supplemented with AMP. PurR may affect many genes; any competence genes whose expression is substantially increased by the knockout will be targeted for direct investigation. Examination of the expression time courses described above may reveal whether genes with PurR boxes are normally repressed in sBHI, and the extent to which repression is lifted as the medium becomes depleted or after cells are transferred to M-IV. This will indicate whether nucleotide pools limit competence development in rich medium. If the results warrant, we will directly compare *purR*⁺ and *purR*⁻ gene expression during exponential growth in sBHI. (*Kenith Chan, a PhD student, will carry out this project.*)

2. Do nucleotide pools control (i) cAMP levels? (ii) *sxy* expression?

Purine-repressed transcription from *comA-F* is independent of PurR and is restored by high concentrations of cAMP (Fig. 7 in #4). One explanation, that purines affect cAMP levels and thus all CRP/cAMP-regulated genes, can be tested by a PatSearch search for CRP sites and microarray comparisons of *cya* and wildtype cells in M-IV and of cells in M-IV with and without GMP. If the latter shows that only competence genes are affected, purines may instead act by decreasing Sxy. Western blots with an anti-Sxy antibody will show whether less Sxy is produced when nucleotides are added, and whether *sxy* hypercompetence mutations make expression less sensitive to nucleotide supplementation. The microarray time courses may show whether increases in *sxy* translation correlate with depletion of intracellular nucleotide pools (indicated by induction of PurR-repressed genes). If effects on Sxy are mediated by post-transcriptional regulation acting via the *sxy* mRNA 2° structure, expression of the *sxy* protein fusion should be sensitive to GMP. The effect of GMP should be reduced in those protein fusions with mutations that weaken the mRNA secondary structure (*sxy-I*) or eliminate it (*sxy-II*). Any nucleotide effects on *sxy* expression should be independent of PurR.

We will take advantage of the genome sequences of *P. multocida* and *A. actino* to solidify and extend our understanding of the regulation of *sxy*. For example, we will examine the *sxy* 5' regions for evidence of secondary structure like that found in *H. influenzae sxy* mRNA. Such evidence would reinforce the hypothesis that the base pairing has regulatory significance. (*This project is suitable for a M.Sc. student, and may develop into a Ph.D. project.*)

3. Does Sxy (i) control transcription of all CRE genes? (ii) control transcription of any non-CRE genes? (iii) act only with CRP/cAMP?

Our working hypothesis is that competence-inducing conditions raise intracellular levels of cAMP and Sxy, and that CRP and Sxy then bind at CRE sites and induce transcription. The CRE sequence was discovered not by functional analysis but by visual inspection of four known competence gene promoters. Searching for matches to a consensus based on the first four identified five more^{33,34}; several have roles in competence⁶⁷. PatSearch will allow the search criteria to be more accurately specified, as a weighted matrix of this larger set rather than a simple consensus. Once all the *H. influenzae* genes with potential CRE sites have been identified, microarrays will be used to compare gene expression in M-IV-induced cultures of *sxy*⁺ versus *sxy*⁻ strains, and in exponentially growing cultures of *sxy*⁺ versus the hypercompetent *sxy-I* mutant. Special attention will be paid to the *ssb*, *isn* and *phpG* genes. These CRE genes are not known to play any specific role in competence (nontransformability of *isn* may result from its deep-rough phenotype (E. R. Moxon, personal communication)), but such a role is not implausible. Question

(iii) will be addressed by microarrays comparing gene expression by sxy^- and sxy^+ strains in a crp^- (or cya^-) genetic background. If Sxy acts only with CRP, and only in the presence of cAMP, their M-IV-induced expression patterns should be identical. (*The technician and/or a graduate student will carry out this research.*)

4. How do Sxy and CRP control transcription from CRE promoters?

Bandshift analysis, Western blotting and site-directed mutagenesis will be used to distinguish between two hypotheses: **I.** CRE sites are a special case of CRP sites and the sequence differences create a need for Sxy to help CRP activate transcription. **II.** CRE sites are ordinary CRP sites and a separate sequence element creates the need for Sxy. Hypothesis I predicts that both Sxy and CRP will bind to CRP sites. Hypothesis II predicts that site-directed mutagenesis of CRE and CRP sites will be unable to change or create a requirement for Sxy. We will focus on regulation acting at the *comA-F* and *rec-2* promoters.

a. Does CRP bind to CRE promoters in the absence of Sxy? We will use bandshift assays to look for binding of CRP to DNA fragments containing CRE sites. **Table 1** gives the details of the bandshift experiments to be done and their interpretation. Initial control experiments are in progress (**assays 1-5**). These use a DNA fragment containing a CRP site and extracts from wildtype cells and *crp* and *cya* mutants, and will allow optimization of assay conditions. **Assays 6 and 7**, of sxy^- cell extracts without and with added cAMP binding to a DNA fragment containing a CRE site, will show whether CRP binds to CRE sites in the absence of Sxy. Extracts from *cya^-* and *crp^-* cells, and Western blotting with anti-CRP antibody will confirm the results.

b. Does Sxy bind to CRE promoters in the absence of CRP? This will be answered by **assay 8**, using extracts from the Sxy-overexpressing mutant *sxy-1* without added cAMP. Bandshifting will mean that Sxy can bind to the CRE in the absence of active CRP. A control will use *sxy-1 crp^-* extracts to test for dependence on inactive CRP (without cAMP), and comigration of Sxy with the shifted band will be confirmed by Western blotting using anti-Sxy antibody.

c. Do CRP and/or Sxy bind to CRE promoters only when both are present? **Assay 9** uses wildtype cell extracts with cAMP. If neither Sxy nor CRP binds alone in assays 7 and 8, a bandshift here (and confirmatory Western) will indicate that binding requires both regulators. If each binds without the other, the final bandshift and Western analyses will show whether both proteins bind when both are present.

Mutagenesis experiments will reveal whether the CRE sequence creates the need for Sxy. CRP and CRE sites differ at the central bases in the highly-conserved symmetric 5-mers, indicated by * in **Fig. 5** (T:A in CRP and C:G in CRE). In *E. coli* CRP sites these bases are the site of the DNA bending responsible for promoter activation⁶⁸. We will test whether putting C:G into CRP sites makes them Sxy dependent and whether putting T:A into CRE sites makes them Sxy independent. If not, the flanking stretches of As and Ts will be reversed, both separately and in combination with the central C:G/A:T changes. If no changes create or eliminate a need for Sxy, deletion analysis will be used to examine other sequences in CRE promoters. DNA bending is essential for CRP activation at its normal promoters, but analysis of protein-induced DNA bending is technically complex. If our bandshift and immunoblot experiments confirm that CRP binds at CRE promoters we will enter into a collaboration with experts in CRP binding and DNA bending.

Andrew Cameron has found CRE sites at some *P. multocida* competence genes (*comA-F*, *rec-2*, *dprA*, *pilA*); we will extend the PatSearch analysis of CRE sites to both *P. multocida* and *A. actino*. Finding CRE sites in competence gene promoters of all three sequenced Pasteurellaceae would strengthen the case for their functional significance. We may also undertake cross-species

complementation experiments by introducing into *H. influenzae* a *P. multocida* or *A. actino sxy* gene on a plasmid, and then knocking out the chromosomal *sxy* gene by transformation with DNA from our *sxy*-knockout mutant. (This is necessary because the *sxy* mutant cannot become competent). If the bandshifts suggest that Sxy interacts with CRP, complementation may require presence of the heterologous *crp* genes, which we can clone by PCR from the respective genomes.

We will also look for CRE pattern matches in *E. coli* K-12 and O157:H7 genomes. *E. coli* has homologs of a number of *H. influenzae* competence genes including *sxy*, *comA-F* and *comJ*⁴¹. Cross-complementation between the *H. influenzae* and *E. coli* *sxy* genes will be tested in collaboration with Steve Finkel (*letter attached*). We have both the *E. coli* and *H. influenzae* *crp* genes cloned in shuttle plasmids, in case complementation requires conspecific CRP. (*Andrew Cameron, a PhD student, will carry out this project.*)

5. How does *murE* control transcription from CRE promoters?

Our initial hypotheses have been unable to explain how the *murE*₇₄₉ mutation causes hypercompetence, so microarray comparisons of gene expression in *murE*⁺ and *murE*₇₄₉ cells in exponential growth will be used to investigate effects on both murein-metabolism genes and competence genes. If, for example, we find that *murE*₇₄₉ causes overexpression of the *mra* operon, we will test whether overexpressing specific genes such as *ftsQ*, *ftsA* or *ftsZ* enhances competence (see **Fig. 6**). If we find that *murE*₇₄₉ causes the same changes in gene expression as the *sxy-1* overexpression mutant, *sxy* protein fusions and anti-Sxy antibody will be used to clarify whether it acts by increasing levels of Sxy. If instead we find that all genes with CRP and CRE promoters are induced, we will examine intracellular cAMP levels. (*The technician and an M.Sc. student will carry out this project.*)

6. Why are some *H. influenzae* isolates not competent?

A number of *H. influenzae* isolates cannot be transformed in the laboratory. This could be because the inducing treatments that work for Rd are inappropriate for these strains, or because they are genetically incapable of developing competence or recombining DNA. Because genetic information about non-competent isolates is scarce, we will begin by using microarrays for a gene survey, asking whether any of the known Rd competence genes are missing in other strains. These experiments investigate gene presence rather than gene expression, so Klenow polymerase will be used to label total genomic DNAs using random hexamer primers. To increase sensitivity, competitions will use a several-fold excess of non-Rd DNA over Rd DNA.

Ideally strains whose transformability and genetic relatedness are both known should be used, to avoid being misled by similarities between closely-related strains. We only know of one such strain – a non-transformable isolate studied by Simon Kroll, which falls in Division II of the serotype *b* strains⁶. We would like to analyze several other non-transformable and transformable isolates, and are investigating whether population genetics studies have been done on the other isolates of known transformability in our collection (six non-transformable and two transformable obtained from Eric Hansen). If time permits we will analyze all the genetic differences (not just competence genes) and look for patterns of shared loss and retention.

If genes are missing, the sequences at their expected locations will be examined for evidence of how deletion occurred. If one or more non-transformable strains contain all the expected competence genes, microarrays will be used to determine whether these genes are induced after exposure to MIV medium. We will initially use only a single time point for each isolate, at a time determined by the initial time course experiments. Standard genetic tools will then be used to investigate any missing or inappropriately expressed genes. If the aberrant pattern of competence gene expression suggests, regulatory genes will also be characterized. Complementation tests

with Rd genes can be used to test specific hypotheses. Because genetic manipulation of nontransformable strains is difficult, we will begin by replacing the Rd homolog with the allele from the nontransformable strain. (*Post-doctoral fellows will carry out this project.*)

Other questions to be addressed as time permits:

gcvA: A *gcvA* knockout has a modest effect on competence; its regulatory targets in *H. influenzae* are unknown. George Stauffer's lab has been using *lacZ* fusions to search for additional GcvA-regulated genes in *E. coli* (personal communication); we will examine the effect of *murE* and *gcvA* mutations on any *H. influenzae* homologs of such genes.

trmE, rpoBC and fis mutations: Insertion mutations in *trmE*, *rpoBC* and *fis* all cause decreases in competence that are disproportionate to their modest growth defects. Microarray analysis will allow determination of the relative strength of effects on competence and on other cellular processes. If some effects are specific to competence, Western blots and protein fusions will be used to find out whether changes in Sxy protein levels are responsible. The *trmE* analysis will have general significance; TrmE is both highly conserved and essential in many bacteria, but its function is not understood^{53,54}. The microarray data will be analyzed for evidence of specific effects in non-competent cells. We have a number of *trmE* insertions at different sites and with different effects on competence⁵².

com753 mutation: One hypercompetence mutation remains unmapped (*com753*). We know this mutation increases expression of *comA-F* and *rec-2*, but preliminary linkage and sequence analysis indicated that it is not in *sxy* or *murE*. Microarray comparisons will reveal whether it acts by a different pathway than our known hypercompetence mutants. If so we will look for the mutation in any genes whose expression it alters or eliminates.

Significance:

Why use microarrays? It would be technically possible to obtain most of the information we seek by gene-specific methods. However the availability of abundant *H. influenzae* whole-genome microarrays at nominal cost permits this faster and more economical approach. Microarray analysis does not obviate the need for research personnel, but more work will be done by the brain and less at the bench. A common concern about microarray work is that the mass of data can be overwhelming, especially if objectives are ill defined. Our microarray analyses will not be 'fishing expeditions'. Rather each analysis tests one or more clear hypotheses, with well-defined questions about the roles of specific genes. The results will direct attention to the best targets for gene-specific experiments. One advantage of using microarrays is that after the initial tightly focused analysis we can also return to the data set with new questions raised by the answer to the original question.

The analyses we propose, using both microarrays and gene-specific methods, will address the critical points of the model shown in Fig. 3. They should reveal the mechanisms by which nucleotides regulate the expression of competence genes, the roles of Sxy and CRP at CRE-containing promoters, and the mechanism by which mutations in the peptidoglycan-biosynthesis gene *murE* stimulate competence. Our understanding of competence in *H. influenzae* is based almost entirely on experience with one strain, Rd. Comparison between different strains of other species has revealed the narrowness of single-strain perspectives⁶⁹. Thus the survey of competence genes and competence gene expression in other transformable and nontransformable isolates should greatly increase our understanding of the regulation and function of DNA uptake. [Note that the lack of recent citations to other lab's work on the regulation of competence in *H. influenzae* is because no other labs are working on this problem. If we don't do it, nobody will.]

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