

# Sigma X induces competence gene expression in *Streptococcus pyogenes*

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## Abstract

Although not thought to become competent for DNA uptake, the bacterium *Streptococcus pyogenes* appears to encode within its genome the DNA uptake and recombination machinery required for competence. The promoters of these genes contain a conserved sequence, CIN-box, which is recognized by the *S. pyogenes* alternative RNA polymerase sigma factor X. Using microarray technology, we found that sigma X induced the expression of competence genes in *S. pyogenes*. Real-time RT-PCR was performed to confirm that the expression of these transcripts was induced 2–265-fold by sigma X. Of the eight CIN-box loci induced, *femB*, the ortholog a virulence factor in *Staphylococcus aureus*, was shown to be transcribed in vitro by RNA polymerase containing sigma X, indicating that sigma X directly activates expression of this CIN-box gene. Sigma X-dependent induction of genes encoding competence machinery in *S. pyogenes* raises the possibility that some strains of this human pathogen can develop competence for genetic transformation.

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**Keywords:** Competence; RNA polymerase; Sigma factor

## 1. Introduction

*Streptococcus pyogenes* (Group A *Streptococcus*, GAS) is a human-specific pathogen that causes a wide spectrum of diseases [7] ranging from self-limiting, localized infections (e.g., impetigo and pharyngitis) to more serious, life-threatening illnesses (e.g., toxic-shock like syndrome and necrotizing fasciitis) [9]. The organism has evolved elaborate regulatory mechanisms for controlling virulence in response to environmental changes. The regulatory systems that have been studied include Mga (multiple gene regulator), RALP (RofA-like protein), Rgg (RofB) and four two-component systems (CovRS, FasBCAX, Ihk/Irr and SilAB) (reviewed in [17]). However, biological functions of numerous putative regulators in GAS remain to be established.

Alternative sigma factors of RNA polymerase play important roles in regulating bacterial gene expression under diverse environmental conditions [15]. In *Streptococcus pneumoniae*, alternative sigma factor, ComX, regulates development of competence for genetic transformation [21]. In addition to com-

petence development, ComX of *Streptococcus mutans* is also involved in biofilm formation and stress tolerance [1]. Competence development in *S. pneumoniae* is signaled by a quorum-sensing system that consists of three components: a signal peptide and a two-component regulatory system composed of a membrane-bound histidine kinase sensor and an intracellular response regulator [28,36]. Competence is induced through quorum-sensing when the cells reach a critical density to where the concentration of the signal peptide reaches a sufficiently high enough level to bind the membrane-bound receptor. Following activation, the response regulator initiates transcription of genes including ComX that are required for expression of “late” competence genes [22]. These “late” competence genes encode proteins required for binding and uptake of exogenous DNA into the recipient cell [16,30]. Some of them also may be required for virulence [6].

ComX is encoded by identical, duplicate genes in the *S. pneumoniae* genome [19]. ComX recognizes a specific DNA sequence in promoters, called a CIN-box, [6]. Approximately 15 genes have been identified through both genomic searches and microarray analyses that contain a CIN-box promoter [29]. Putative orthologs of the *comX* genes of *S. pneumoniae* have recently been identified in *S. pyogenes* [27]. Their open read-

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ing frames encode proteins that are 40% identical at the amino acid level to ComX. This ComX homologue, named as SigX in GAS, functions as an alternative sigma factor and directs transcription of several CIN-box-containing promoters [27,28]. The activity of SigX is negatively regulated by ClpP-mediated proteolysis. Studies in our laboratory have failed to reveal a role for SigX of GAS in bacterial virulence, stress tolerance or biofilm formation (unpublished data). GAS are not known to develop competence for DNA uptake; however, to investigate the role of SigX of GAS we conducted genomic searches for homologous competence genes and examined whether these genes are expressed in response to SigX.

## 2. Materials and methods

### 2.1. Bacterial growth conditions and media

GAS strain JRS4 is a streptomycin derivative of D471, an M-type 6 strain [32]. Unless otherwise detailed, GAS strains were grown at 37 °C without agitation in Todd Hewitt broth supplemented with 0.2% yeast extract (THY). Antibiotics were used at the following concentrations: chloramphenicol, 5 µg/ml for GAS and 20 µg/ml for *E. coli*; erythromycin, 0.5 µg/ml for GAS and 250 µg/ml for *E. coli*; kanamycin, 200 µg/ml for GAS and 50 µg/ml for *E. coli*; and spectinomycin, 100 µg/ml for GAS and 100 µg/ml for *E. coli*.

### 2.2. Microarray construction

The GAS microarrays consist of 2328 unique 70-mer oligonucleotide probes designed and synthesized by Operon Technologies (Valencia, CA) using a proprietary algorithm to target unique, non-repetitive reading frames present in three sequenced genomes (TIGR annotation as of December 02, 2002). 1932 oligonucleotides of a strain of serotype M1 (SF370), 165 probes of a strain of serotype M3 (MGAS315), and 231 probes of a serotype M18 (MGAS8232). The nucleotide design and synthesis was performed in collaboration with Dr. Kevin McIver (UT Southwestern Medical Center, Dallas, TX). Oligonucleotides were spotted in triplicate onto UltraGAPS2 microarray slides (Corning #40017) by Microarrays, Inc. (Nashville, TN). Slides were rehydrated, crosslinked, and blocked in prehybridization solution (50% formamide, 5× SSC, 0.1% SDS, 0.1 mg/ml bovine serum albumin) according to manufacturer's instructions (Corning).

### 2.3. cDNA labeling and hybridization conditions

Total RNA was isolated as previously described [23]. cDNA was transcribed and labeled from RNA incorporating aminoalloyl-dUTP (Fairplay microarray labeling kit, Stratagene #252003) using 3 µg random hexamers (Invitrogen #48190-011) per reaction. Cy3 and Cy5 monoreactive dyes (Amersham #PA23001 and #PA25001) were used to directly label the cDNA according to the manufacturer's specifications. The labeled cDNA was purified using QiaQuick PCR purification columns (Qiagen #28104) and concentrated using Microcon YM30 columns (Amicon #42410). The incorporated

label amount was measured and quantified using absorbance at 550 nm (Cy3) or 650 nm (Cy5).

For each set of microarray experiments, a reference cDNA sample consisted of pooled Cy5-labeled cDNA generated from all RNA samples to be examined. The test samples consisted of cDNA generated from RNA from each sample that was labeled with Cy3 in triplicate and pooled prior to hybridization. Therefore, the wild type control arrays for the experiment consisted of Cy5-labeled reference cDNA (pooled wild type and overexpression cDNA samples) and Cy3-labeled test cDNA (wild type cDNA labeled in triplicate and pooled). Each array consisted of an aliquot of reference cDNA and test cDNA containing 60 pmol each Cy-Dye was mixed with 60 µl hybridization buffer 1 (Ambion #8861), heated for 5 min at 95 °C, centrifuged at 12 000 g for 2 min prior to being applied to the array. Each sample probe was allowed to hybridize overnight in a 42 °C water bath (at least 16 h). Following hybridization, arrays were washed once with 2× SSC, 0.1% SDS and twice with 0.1× SSC.

### 2.4. Microarray data analysis

Following washing, arrays were scanned using a GenePix 400A scanner (Axion, Inc.). Resulting images were visually inspected and analyzed using GenePix Pro 3.0 software. For each of the hybridizations, signals recorded as below a threshold level (the mean background level of a whole experiment + 1 SD) were set to threshold level to avoid potentially erroneous expression ratios. Data for each gene from each of three individual spots on the array were averaged and the value represented a single value for the experiment. The data was filtered to remove values for genes that were not present in the JRS4 strain; genes from the M3 and M18 genomes. Expression values for each gene were then expressed as a fraction of the total amount of expression on each array (using Global Normalization), in order to make comparisons between arrays valid. Normalized data was then log<sub>2</sub>-transformed and statistically analyzed using Significance Analysis of Microarrays (SAM) software [35].

### 2.5. Isolation of total RNA and real time RT-PCR analysis

JOS34 ( $\Delta clpP$ ) [26] and JOS37 (JOS34 [pJO162]) [26] strains were grown at 37 °C in regular THY medium to exponential phase ( $OD_{600} \approx 0.7$ ). RNA was immediately stabilized by adding 2 volumes of RNAProtect™ bacterial reagent to cell cultures. Cells from 2 ml of cell culture were harvested and resuspended in 300 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 20 mg/ml lysozyme. After incubating in a shaker for 10 min at room temperature, cells were transferred to lysing matrix B tubes (QBiogene) containing 300 µl of 2× tissue and cell lysis buffer (Epicentre Technologies) and lysed using FastPrep FP120 beads beater (QBiogene). Total cellular RNA was subsequently prepared using the MasterPur™ RNA purification kit (Epicentre Technologies), with two DNase I digestion steps to remove DNA from the RNA preparations. Quality and integrity of total RNA was assessed on 1% formaldehyde-agarose gels. The steady levels of

Table 1  
Competence genes in *B. subtilis*, *S. pneumoniae*, and *S. pyogenes*

Function	<i>B. subtilis</i>	<i>S. pneumoniae</i> R6	<i>S. pyogenes</i> M1
Quorum-sensing			
Pheromone/competence signaling peptide	<i>comX</i>	<i>comC</i>	–
Export system	<i>comQ</i>	<i>comA, comB</i>	–
Histidine-kinase response regulator	<i>comA, comP</i>	<i>comD, comE</i>	–
Transcription and transcriptional control			
Competence transcription factor	<i>comK</i>	<i>comX</i>	<i>comX</i> (Spy0300)
Positive regulators	<i>sinR, med</i>	<i>comW</i>	–
Negative regulators	<i>mecA</i>	<i>ciaR/H; mecA</i>	<i>ciaR/H; mecA</i> (Spy1237-6; Spy0281)
Late competence transcription	–	* <i>coiA</i>	* <i>coiA</i> (Spy1395)
DNA binding and uptake systems			
DNA binding and uptake	<i>comEA–EC</i>	* <i>celA, celB</i>	* <i>comEA, comEC</i> (Spy1409-8)
DNA binding and transport	<i>comGA–GG</i>	* <i>cglA–cglE</i>	* <i>comYA–YD</i> (Spy0101-6)
DNA uptake	<i>comFA–FC</i>	<i>comFA, comFC</i>	<i>comFA, comFC</i> (Spy1616-5)
DNA processing and recombination			
DNA processing	<i>smf</i>	* <i>smf</i>	* <i>smf</i> (Spy1163)
DNA repair and recombination	<i>recA</i>	* <i>recA, *ssb2</i>	* <i>recA, *ssb2</i> (** NA, Spy2116)
Competence-damage inducible protein	<i>cinA</i>	* <i>cinA</i>	* <i>cinA</i> (Spy2117)
Signal peptidase	<i>lspA</i>	<i>lspA</i>	<i>lspA</i> (Spy0826)

\* CIN-box genes.

\*\* NA = not annotated.

interested genes were determined by real-time quantitative RT-PCR analyses using iScript one-step RT-PCR Kit with SYBR Green and the iCycler iQ real-time system (Bio-Rad) under the following conditions: 65 °C—5 min, 48 °C—10 min, 95 °C—5 min (95 °C—10 s, 52 °C—30 s) for 45 cycles. Reactions for each sample were performed in triplicate using 50 ng RNA template. A reaction without reverse transcriptase was included for each sample, which served as a control for DNA contamination. The difference in cycle threshold (CT) between RNA samples from JOS34 and JOS37 strains was calculated. The transcript level of SigX-independent *emm* gene was also determined as a control of CT variation from the two RNA samples. The resulting  $\Delta$ CT values for *emm* were subtracted as background corrections from the  $\Delta$ CT values for the interested genes, generating  $\Delta\Delta$ CT [20]. Fold difference was calculated according to the equation  $2^{\Delta\Delta CT}$ . The PCR product identities were confirmed by electrophoresis on 1.5% agarose gels with ethidium bromide staining and product uniformity was determined using melting curves (iCycler Instruction Manual, Bio-Rad).

### 2.6. In vitro transcription assays

In vitro transcription reactions were performed as described previously [20]. Plasmid pJO92 [27] was digested with *Hind*III and used as template DNA. Core RNA polymerase was preincubated on ice in 1 × transcription buffer with 0.2 μg of purified  $\sigma^A$  or  $\sigma^X$  on ice to allow for association. It was then incubated with the *femB* promoter. Transcription was initiated by adding ribonucleotides, with the UTP labeled at the  $\alpha$  position with  $^{32}$ P. The radiolabeled transcripts were subjected to electrophoresis on a 6% agarose gel containing 7 M urea, and the resulting gel was exposed to autoradiography.

## 3. Results

### 3.1. *S. pyogenes* encodes genes homologous to those required for competence in *B. subtilis* and *S. pneumoniae*

We searched the genome for homologues to known competence genes in *S. pneumoniae* and another Gram-positive bacterium, *B. subtilis* in which competence development has been well characterized [14,25]. Our primary focus was on identifying sets of genes involved in DNA uptake, binding, and recombination, as well as a putative quorum-sensing system for initiation of competence. Analyses of the GAS genome indicated that the “late” competence genes, such as *recA*, *cinA* and *smf* are present; moreover, genes involved in DNA binding, uptake, and recombination are conserved across the three species (Table 1) [10]. Many of these genes also contain the conserved CIN-box sequence, which is recognized by ComX in pneumococcus. Additionally, known regulators of competence such as *mecA* and the two-component system *ciaH/ciaR* are present in GAS [10].

The genetic organization of the genes encoding proteins required for DNA uptake and binding exhibit differences among the *Streptococcus* and naturally competent *Bacillus* species (Table 1). The *comEA–comEC* operon exists as one transcriptional unit in *Bacillus subtilis*; however in these streptococcal species, the *comEC* homologues are encoded in separate operons. In pneumococcus and GAS, the *comGA–comGG* system that assists in DNA transport across the peptidoglycan, lacks homologues to *comGE*, *comGF* and *comGG*. The streptococci are similar also in their lack of *comFB* from the DNA uptake system *comFA–comFC* of *B. subtilis* [10,14,25]. Evidently *S. pneumoniae* does not require ComFB for DNA uptake.

Table 2  
CIN-box-containing genes in *S. pyogenes*

Locus <sup>a</sup>	Gene(s) (putative function)
Spy0101–Spy0107	<i>comYA, comYB, comYC, Spy0104–Spy0107</i> (competence)
Upstream of Spy0121	<i>ssb2</i> (recombination)
Spy0235	<i>dut</i> (dUTPase)
Spy0571	<i>licT</i> (transcriptional antiterminator)
Spy0713	<i>pepD</i> (dipeptidase)
Spy0816–Spy0818	<i>nifS.1</i> (iron sulfur cofactor)
Spy1155	<i>Spy1155</i> (hypothetical)
Spy1163	<i>smf</i> (competence)
Spy1205	<i>femB</i> (peptidoglycan crosslinking)
Spy1245–Spy1240	<i>pstS, pstC2, pstC, pstB2, pstB</i> (phosphate transport)
Spy1395	<i>coiA, pepB</i> (competence)
Spy1409–Spy1408	<i>comEA, comEC</i> (competence)
Spy1422	<i>recR</i> (recombination)
Spy1735–Spy1733	<i>Spy1735</i> (hypothetical)
Spy2117–Spy2116	<i>cinA, recA</i> (competence/recombination)

<sup>a</sup> Genomic loci are based on the genome of the M1 strain of *S. pyogenes* SF370 (Ferretti et al., 2001).

### 3.2. SigX induces expression of competence genes in GAS

SigX has been shown to interact with RNA polymerase and direct transcription from three CIN-box promoters from GAS [27]. Scans of the GAS genome reveal approximately 16 potential CIN-box loci, and several of those are homologues to known competence genes in *B. subtilis* and *S. pneumoniae* (Table 2). In addition to CIN-box genes, there may be non-CIN-box genes either directly, or indirectly regulated by SigX that are involved in competence. Therefore, we used an oligonucleotide array to identify gene transcripts affected by SigX. The conditions under which SigX is expressed are unknown. However, Opdyke et al. [26] showed that transcripts from three CIN-box genes are produced in a GAS strain that is mutated for *clpP* and in which *sigX* is placed downstream from a heterologous promoter. Therefore, we investigated the transcriptional effects of overexpressing *sigX* in a similar strain.

To identify gene transcripts affected by SigX, we compared transcript levels in a *sigX* overexpression strain lacking *clpP* with those from a strain lacking only *clpP*. These strains were grown at 37 °C to early log phase in THY broth, and RNA was harvested and used to generate cDNA probes that were hybridized to two array slides. Transcripts from 172 genes appeared to be elevated or repressed by SigX at least 2-fold. These included genes homologous to those involved in competence from other bacteria (supplementary Table S1, and Table 3).

The microarray results for several CIN-box competence genes were confirmed by real-time RT-PCR using newly isolated RNA (Table 3). Quantitation of the transcript levels indicated a substantial increase in transcription levels, with a minimum expression level of 12.1-fold for *coiA*, to a maximal expression of 265-fold for the *comYA* operon.

### 3.3. SigX directs femB

Of the 172 transcripts demonstrated to be either activated or repressed in response to SigX overexpression, eight loci evi-

Table 3  
Expression of CIN-box genes in a SigX overexpression strain versus a *clpP* mutant strain

Locus <sup>a</sup>	Gene(s) (putative function)	Expression RT-PCR <sup>b</sup>
Spy0101–Spy0107	<i>comYA–C, Spy0104–7</i> (competence)	265.0 ± 1.1
Upstream of Spy0121	<i>ssb2</i> (recombination)	222.9 ± 1.2
Spy1163	<i>smf</i> (competence)	25.8 ± 2.2
Spy1205	<i>femB</i> (peptidoglycan crosslinking)	20.7 ± 1.3
Spy1395	<i>coiA, pepB</i> (competence)	12.1 ± 1.4
Spy1409–Spy1408	<i>comEA, comEC</i> (competence)	15.5 ± 1.3
Spy2117–Spy2116	<i>cinA, recA</i> (recombination)	39.1 ± 1.2

<sup>a</sup> Genomic loci are based on the genome of *S. pyogenes* SF370 (Ferretti et al., 2001).

<sup>b</sup> Fold change in expression versus wildtype culture as determined by real-time RT-PCR.

dently have conserved CIN-box sequences, which may indicate that RNA polymerase containing sigma X directly activates their expression. We tested this hypothesis for one of the CIN-box-containing genes, *femB*, in an in vitro transcription experiment because this promoter has a perfect CIN-box, and Opdyke et al. [27] had previously shown evidence that this promoter could be used by RNA polymerase containing sigma X. The transcript of this gene was elevated over 20-fold in vivo by overexpression of SigX (Table 3). Therefore, core RNA polymerase was preincubated with either  $\sigma^A$  or  $\sigma^X$ , and then incubated with a DNA template containing the *femB* promoter. Transcription was initiated by the addition of ribonucleoside triphosphates, one of which was labeled at the  $\alpha$  position with <sup>32</sup>P. Core RNA polymerase preincubated with  $\sigma^X$  produced a specific transcript from the *femB* promoter (Fig. 1). This transcript was not detected when RNA polymerase was preincubated with  $\sigma^A$ , or when the transcript reaction was carried out with core RNA polymerase lacking a sigma factor (Fig. 1).

## 4. Discussion

In this study, we have identified *S. pyogenes* competence loci that are activated by induction of sigma X. Examination of the GAS genomes indicate fifteen open reading frames whose upstream regions contain a CIN-box [2,10,33]. Of these fifteen, nine were detected as activated by SigX in this study. The majority of these genes are potentially involved in competence development. Transcription of genes at six loci that have strong homologies to competence genes of pneumococcus were induced in response to sigma X from 2-fold to 265-fold. These included genes encoding proteins involved in DNA uptake and recombination, such as *ssb2*, the *comYA* locus, and *smf*. Transcription of these genes was confirmed using real-time RT-PCR, although we found quantitative differences between the array and real-time PCR results. The lower fold activations seen by microarrays may be due to the inherent differences in sensitivity between the technique and real time RT-PCR. Specifically, the array used was constructed with relatively short oligopeptides representative of GAS genes, which may limit the sensitivity of detection. The dampening of signal from the microarrays may also be related to low levels of non-specific hybridization,

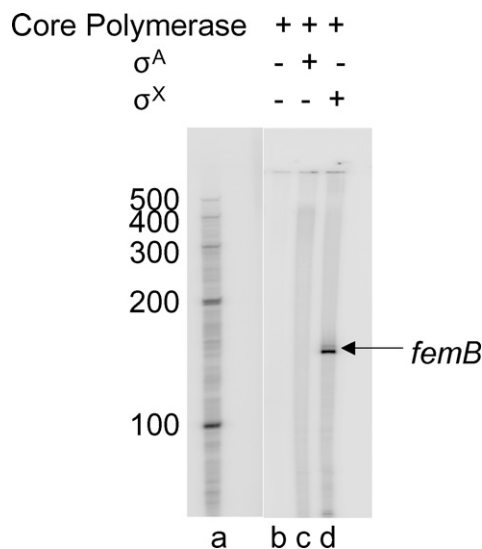


Fig. 1. Sigma-X-directed in vitro transcription of *femB*. Radiolabeled molecular weight size markers are indicated to the left of the gel (lane a). Core RNA polymerase was preincubated alone (lane b), or with either  $\sigma^A$  (lane c) or  $\sigma^X$  (lane d) and then incubated with the *femB* promoter. Transcript was initiated by the addition of ribonucleoside triphosphates labeled with  $^{32}\text{P}$  as described in Section 2. Shown is a phosphorimage of the transcripts after electrophoresis into a 6% polyacrylamide gel containing 7 M urea. The region of the original gel that separated lanes a and b has been deleted. The position of the *femB* transcript is indicated by the arrow.

or cross-hybridization, causing a higher overall quantitation of basal expression levels [31,35]. The dynamic range of DNA microarrays is much lower than that of real time RT-PCR, suggesting that they may be improved by optimizing the design and hybridization chemistry of the assays [35]. Moreover, the sequences of the oligonucleotides used to build the microarray were based on a sequenced reference strain, which contains differences from the strain that we used to produce the RNA. In contrast, the oligonucleotides used for the real-time RT PCR were perfectly complementary to the RNA. Therefore, these differences may have affected the quantitative results of the microarray data. It is also unknown whether technical issues with the array prevented detection of expression from several putative CIN-box genes, or whether expression of these genes is regulated by sequences or factors in addition to the CIN-boxes.

Our observation that competence genes such as *cinA*, *comEA*, and *coiA* are expressed in response to sigma X in GAS raises the question of why this strain apparently does not develop competence for transformation. The answer may in part be that the strain lacks the quorum-signaling system that would induce *sigX* expression. No homologue to the *comABCDE* quorum-signaling system of pneumococcus is present in the majority of sequenced GAS strains [10,13,33]. The presence of DNA uptake machinery in GAS may be evidence of a yet undiscovered, novel regulatory circuit for the activation of competence.

There is some evidence to suggest the possibility that some strains of *S. pyogenes* may be naturally transformable. A unique locus encoding a set of genes homologous to the pneumococcus *comABCDE*, called *silABCDE*, is present in an M18 strain [12]. The putative quorum-sensing peptide component of the

*silABCDE* locus, *silC*, is preceded by a CIN-box promoter. Moreover, C. Hidalgo-Grass et al. [14] found evidence for genetic transmission in mixed culture. This study did not examine whether *sigX* was involved. However, these results indicate that transformable strains of GAS may exist.

In addition to putative competence gene expression of sigma X induced *femB*, a virulence factor in *S. aureus* [4]. *femB* has a perfect CIN-box, and we found that its promoter is used in vitro by RNA polymerase containing sigma X. The *fem* genes appear to encode enzymes essential for cross-linking cell walls, and involved in methicillin resistance [3,18]. In *S. aureus* *femB* mutants have reduced cell wall cross-linking and therefore, a reduced sensitivity to methicillin and other antibiotics [4,34]. The role of FemB in remodeling of peptidoglycan during competence in *S. pneumoniae* has not been examined, and its role in *S. pyogenes* is unknown. *femB* has been determined to be a virulence factor in *S. aureus* using signature-tagged mutagenesis in a mouse model [24].

Recently, along with *S. pyogenes*, homologues to DNA uptake and recombination machinery have been detected in other non-transformable bacteria such as *Lactococcus lactis* and *Listeria monocytogenes* [8]. Homologues to the *B. subtilis* transcription factor required for competence development, ComK, has been identified in *L. monocytogenes*, and *L. lactis* have a homologue to ComX of pneumococcus [5,11]. These observations raise the possibility that some strains of these species may be transformable or are just a few evolutionary steps from developing this ability.

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### Supplementary material

Supplementary data associated with this article can be found on ScienceDirect in the online version. Please visit doi: 10.1016/j.resmic.2006.07.002.

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