Inhibition of competence development in *Streptococcus pneumoniae* by increased basal-level expression of the ComDE two-component regulatory system

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Natural competence for genetic transformation in *Streptococcus pneumoniae* is controlled by the ComCDE signal-transduction pathway. Together, ComD, a membrane histidine kinase, and ComE, its cognate response regulator, constitute a typical two-component regulatory system involved in sensing the comC-encoded competence-stimulating peptide (CSP). The comCDE operon is strongly upregulated when CSP reaches a critical threshold, probably to coordinate competence induction throughout the population. During a study of the early regulation of the comCDE operon, a mutation which resulted in increased β-galactosidase production from a comC::lacZ fusion was isolated. This mutation, which was characterized as a G→T change in the transcription terminator of the tRNAArg located immediately upstream of comCDE, is suggested to destabilize the terminator and to allow transcriptional readthrough of comCDE. Here, it is shown that, quite unexpectedly, the mutation confers reduced transformability. A series of experiments undertaken with the aim of understanding this surprising phenotype is described. Evidence is presented that increased basal-level expression of comDE impedes both spontaneous and CSP-induced competence in *S. pneumoniae*. There is a discussion of how an increased concentration of ComD and/or ComE could affect competence development.

INTRODUCTION

Natural competence for genetic transformation in *Streptococcus pneumoniae* is a transient physiological state allowing cells to take up exogenous DNA. The development of competence is controlled by a regulatory cascade involving an unmodified peptide pheromone, the CSP (competence-stimulating peptide), encoded by comC (Håvarstein et al., 1995; Pestova et al., 1996), its dedicated secretion apparatus, ComAB (Hui & Morrison, 1991; Hui et al., 1995), and a two-component regulatory system (TCS), ComDE (Pestova et al., 1996). When CSP accumulates in the medium, it stimulates its receptor, the membrane-bound histidine kinase (HK) ComD. It is assumed that ComD then autophosphorylates and transphosphorylates its cognate response regulator ComE, activating the expression of comAB, comCDE and operons belonging to the so-called early class (for a review, see Claverys & Håvarstein, 2002). ComE boxes, 9 bp imperfect direct repeats separated by a stretch of 12 nucleotides to which ComE binds specifically (Ween et al., 1999), are present in the promoter region of ComE-regulated operons. Expression of the late com genes requires the alternative sigma factor ComX (Lee & Morrison, 1999) and a newly discovered positive regulator of competence termed ComW (Luo et al., 2004; Sung & Morrison, 2005). Recent transcriptome studies have increased the number of known CSP-induced genes from ~47 to 105–124 (Dagkessamanskaia et al., 2004; Peterson et al., 2004). The set of 91 induced genes common to the two studies includes 17 early and 60 late genes (Guiral et al., 2006a). Among the latter, only 14 genes are necessary for transformation; they encode proteins involved in DNA uptake and the processing of internalized ssDNA.

To better understand the early control of competence development, mutants upregulating competence (termed CUP, for competence up) have been isolated using a comC::lacZ transcriptional fusion in a medium non-permissive for spontaneous competence development (Martin et al., 2000). The UP mutation, a G→T change within the putative transcription terminator (ter) of the tRNAArg located immediately upstream of the comCDE

Abbreviations: CEP, chromosomal expression platform; CSP, competence-stimulating peptide; CUP, competence up; HK, histidine kinase; RLU, relative luminescence unit; RT, reduced transformability; TCS, two-component system.

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Fig. 1. Map of the control region of the comCDE operon. (Top) The location of the RT mutation (previously named UP; Martin et al., 2000) within ter, the putative transcription terminator of tRNA Arg, is indicated together with the positions of the ComE binding sites (ComE boxes), the proposed −10 region (Ween et al., 1999) and the +1 site (our unpublished data). (Bottom) Structure of the insertion-duplication generating a comC::luc (comC+) fusion. Vertical thin bars indicate the chromosomal region amplified with primer pair MP120/BM40 (Bergé et al., 2002), cloned in an Escherichia coli replicative vector and duplicated in S. pneumoniae strains through integration of the resulting recombinant plasmids, pR414 (Bergé et al., 2002) and pR428 (Guiral et al., 2005) (Table 1). luc and ant indicate, respectively, the luciferase and antibiotic resistance genes (Ery R for pR414 and Cm R for pR428; Table 1) present in the vector moiety. The latter is not drawn to scale. Note that the RT mutation (indicated in parentheses) is present at each copy of ter in strain R1573 (Table 1).

METHODS

Operon, was identified during this screen (Fig. 1). The UP mutation is suggested to destabilize ter, thus leading to transcriptional readthrough of comC::lacZ (Martin et al., 2000). It was therefore expected that its introduction in front of the comCDE operon would lead to comCDE overexpression and result in competence derepression. Meanwhile, this mutation has been observed to confer an unusual β-haemolytic phenotype on blood agar plates, unique among the CUP mutants. β-Haemolysis has been shown to result from pneumolysin release and to involve the lysis of non-competent cells triggered by competent cells, a phenomenon we refer to as allolysis (Guiral et al., 2005). Here, we show that contrary to expectation and unlike all other CUP mutants, the UP mutation does not derepress competence but reduces transformability. It was therefore renamed RT (for competence but reduces transformability). Here, we show that contrary to expectation and unlike all other CUP mutants, the UP mutation does not derepress competence but reduces transformability. It was therefore renamed RT (for competence but reduces transformability). We report experiments aimed at elucidating this surprising phenotype. We take advantage of CEP (chromosomal expression platform), a newly developed tool for ectopic gene expression in S. pneumoniae (Guiral et al., 2006b), to show that the increased basal level of ComDE mimics both the reduced transformability and the β-haemolytic phenotype of the RT mutant.

Bacterial strains, growth conditions and transformation. Streptococcus pneumoniae strains and plasmids used in this study are described in Table 1. Precompetent cells were grown at 37°C in CAT or C+Y medium (Berge et al., 2002) to OD550 0·4, centrifuged, resuspended in fresh C+Y medium containing 15% glycerol (v/v) and kept frozen at −70°C. Acid C+Y medium (pH 6·9) was routinely used for precompetent cultures, since a low initial pH is known to prevent spontaneous competence development (Chen & Morrison, 1987). To monitor spontaneous competence induction, cells were then gently thawed and aliquots were inoculated in C+Y medium. Transformation was performed as described previously (Martin et al., 2000), using precompetent cells treated at 37°C for 10 min with synthetic CSP1 (100 ng ml⁻¹) to induce competence. After addition of transforming DNA, cells were incubated for 20 min at 30°C. Transformants were selected by plating in 10 ml CAT agar supplemented with 4% horse blood, followed by challenge with a 10 ml overlay containing chloramphenicol (Cm) 9 µg ml⁻¹, erythromycin (Ery) 0·1 µg ml⁻¹, kanamycin (Kan) 500 µg ml⁻¹, spectinomycin (Spc) 200 µg ml⁻¹ or streptomycin (Sm) 400 µg ml⁻¹, after phenotypic expression for 120 min at 37°C.

Construction of CEPcomDE. CEP, a chromosomal expression platform for ectopic, maltose-driven gene expression in S. pneumoniae, has recently been constructed (Guiral et al., 2006b). To place comDE at CEP under the control of PmI, a maltose-inducible promoter, the genes were amplified with the primer pair comDnco/BM93, using as template R800 chromosomal DNA (Table 1). After digestion by Ncol/BamHI, the comDE fragment (2086 bp) was ligated with Ncol/BamHI-digested plasmid pCEP (Table 1). Transformation of strain R1556 with the ligation mixture and selection for KanR transformants was used to generate strain R1564 (CEPcomDE).

Luciferase activity. The Photinus pyralis luc gene, which encodes firefly luciferase, was used as a transcriptional reporter to measure the expression of comCDE or competence. In the latter case, measurement was achieved using a fusion between luc and a gene specifically induced at competence, either comC [where appropriate, i.e. in the absence of the RT mutation (e.g. strain R1568)] or a representative late com gene, sibB (Table 1). A direct correlation between luciferase activity and transformation level was observed under widely varying levels of competence (Berge et al., 2002).
Table 1. Strains, plasmids and primers used in this study

C and A indicate, respectively, the co-transcribed and the reverse orientation of an inserted mini-transposon antibiotic-resistance gene with respect to the targeted gene.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype/description</th>
<th>Source/reference</th>
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<tbody>
<tr>
<td>S. pneumoniae</td>
<td></td>
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<tr>
<td>R6</td>
<td>Non-capsulated D39 derivative</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>R800</td>
<td>R6 derivative</td>
<td>Martin et al. (1985)</td>
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<tr>
<td>R246</td>
<td>R800, but hexA3::ermAM; EryR</td>
<td>Alloing et al. (1998)</td>
</tr>
<tr>
<td>R304</td>
<td>nov1, rif23, str4t; NovR, RifR, SmR</td>
<td>Mortier-Barrière et al. (1998)</td>
</tr>
<tr>
<td>R315</td>
<td>R800, but ∆comCDE; CmR</td>
<td>Alloing et al. (1998)</td>
</tr>
<tr>
<td>R391</td>
<td>R800, but comA::kan; KanR</td>
<td>Guiral et al. (2005)</td>
</tr>
<tr>
<td>R686</td>
<td>R800, but RT-comC::lacZ (pXF520), RT-comC+, comA::ermAM, egf::spc; CmR, EryR, SpcR</td>
<td>Guiral et al. (2005)</td>
</tr>
<tr>
<td>R848</td>
<td>R246, but RT-comC::lacZ (pXF520), RT-comC+; CmR, EryR</td>
<td>This study</td>
</tr>
<tr>
<td>R854</td>
<td>R848, but ::spc190::comA+ (by transformation with R467 chromosomal DNA; Martin et al., 2000); CmR, EryR, SpcR</td>
<td>This study</td>
</tr>
<tr>
<td>R912</td>
<td>R800, but RT-comC::lacZ (pXF520), RT-comC+, ::spc190::comA+; CmR, SpcR</td>
<td>This study</td>
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<td>R1065</td>
<td>R800, but RT, rpsL1; SmR</td>
<td>Sung et al. (2001)</td>
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<td>R1555</td>
<td>R800, but ssbB::luc (pR424), ssbB+, rpsL1; CmR, SmR</td>
<td>Guiral et al. (2005)</td>
</tr>
<tr>
<td>R1556</td>
<td>R800, but ssbB::luc (pR424), ssbB+, rpsL1; CmR, SmR</td>
<td>This study</td>
</tr>
<tr>
<td>R1564</td>
<td>R800, but CEPcomDE [by transformation with a ligation mixture of Ncol/BamHI-digested (pCEP and comDE PCR fragment); see Methods], ssbB::luc (pR424), ssbB+, rpsL1; KanR, CmR, SmR</td>
<td>This study</td>
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<td>R1567</td>
<td>R800, but CEP3 (by transformation with pCEP), ssbB::luc (pR424), ssbB+, rpsL1; KanR, CmR, SmR</td>
<td>Guiral et al. (2006b)</td>
</tr>
<tr>
<td>R1568</td>
<td>R800, but comC::luc (pR414; see Fig. 1, bottom), comC+, comA::kan42A, rpsL1; EryR, KanR, SmR</td>
<td>This study</td>
</tr>
<tr>
<td>R1573</td>
<td>R800, but RT-comC::luc (pR428; see Fig. 1, bottom), RT-comC+, comA::kan42A, rpsL1; CmR, KanR, SmR</td>
<td>This study</td>
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<tr>
<td>Escherichia coli</td>
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<td>LE392</td>
<td>F- c14 (McrA-) hsdR514 (rK mKg) gntV44 supF58 lacY1 galK2 galT22 metB1 trpR55</td>
<td>Sambrook et al. (1989)</td>
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<td>Plasmids</td>
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<td>pR414</td>
<td>CoLE1 (p5.00) derivative; EryR, carries a comC-targeting fragment adjacent to luc; insertion-duplication in S. pneumoniae generates a comC::luc (comC+) fusion</td>
<td>Prudhomme &amp; Claverys (2006)</td>
</tr>
<tr>
<td>pR424</td>
<td>CoLE1 (pEVP3) derivative; CmR, carries an ssbB-targeting fragment adjacent to luc; insertion-duplication in S. pneumoniae generates an ssbB::luc (ssbB+) fusion</td>
<td>Chastanet et al. (2001)</td>
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<tr>
<td>pR428</td>
<td>pR424 derivative; CmR, carries a comC-targeting fragment, with the RT mutation, adjacent to luc; insertion-duplication in S. pneumoniae generates a comC::luc (comC+) fusion (Fig. 1, bottom)</td>
<td>Guiral et al. (2005)</td>
</tr>
<tr>
<td>pCEP</td>
<td>pSC101 derivative; SpcR, KanR, carries the S. pneumoniae chromosomal expression platform, CEP</td>
<td>Guiral et al. (2006b)</td>
</tr>
<tr>
<td>Primers</td>
<td>Sequence*: gene; position†</td>
<td></td>
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<tr>
<td>comDnco</td>
<td>GAAAGAGGcATGGATTTATTGGGATTG; comD; + 138</td>
<td></td>
</tr>
<tr>
<td>BM93</td>
<td>GcgggaTcCTAATTGTAATCATCACTTTGAG; comE; + 2240</td>
<td></td>
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</table>

*Small letters in the sequence indicate nucleotide changes to introduce convenient restriction sites.
†With respect to the ATG of the corresponding gene; + indicates downstream.

Luciferase activity was monitored directly in pneumococcal living cells as previously described (Prudhomme & Claverys, 2006) by measuring luminescence, which reports luciferase activity (expressed in relative luminescence units, RLU), in cultures grown in Corning NBS 96-well (320 μl volume) white plates with clear bottoms incubated at 37 °C for up to 6 h in a Luxcy luminometer (Anthos). The use of clear-bottomed plates allowed the measurement of OD625 in parallel with luminescence.
Detection of ComE. Rabbit antibodies raised against ComE were used in Western analysis to monitor the intracellular level of ComE before and after CSP induction (strains R800 and R1065), or after growth in the presence of maltose for strain R1564 (CEP\textsuperscript{comDE}). To prevent spontaneous competence development as well as induction of the maltose promoter at CEP, cultures were grown in acid C+Y medium (see above) containing 0.3 % (w/v) sucrose. Cells (8 x 10\textsuperscript{6}) were inoculated into 4 ml medium and incubated at 37 °C for ~3 h (up to OD\textsubscript{550} 0.15). For maltose induction, 0.3 % (w/v) maltose was substituted for sucrose. For CSP induction, CSP1 (100 ng ml\textsuperscript{-1}) was added, and cells were incubated for 20 min (final OD\textsubscript{550} 0.25–0.3), pelleted by centrifugation and resuspended in 400 μl SEDS buffer (150 mM NaCl, 15 mM EDTA, 0.02 % SDS, 0.01 % deoxycholate) for cell extract preparation. Samples (12 μl) were subjected to 12 % SDS-PAGE gel electrophoresis, followed by immunoblot transfer, as described previously (Martin et al., 1995). The membrane was incubated for 1 h in TTBS (1 x Tris-buffered saline, pH 7.5, with 0.1 % Tween-20) containing ComE antiserum (1 : 3000 dilution), together with 8 % non-fat dried milk and protein extract (from ~2 x 10\textsuperscript{8} cells) from a comE mutant (strain R315) to reduce non-specific background signals, as described elsewhere (Ween et al., 1999).

RESULTS

The RT mutation results in transcriptional readthrough of \textit{comCDE}

The hypothesis that the RT mutation destabilizes the putative transcription terminator of tRNA\textsuperscript{Ala} (Fig. 1, top) and leads to transcriptional readthrough of \textit{comCDE} (Martin et al., 2000) was checked using a \textit{comC}::\textit{luc} transcriptional fusion (Fig. 1, bottom). Luciferase activities were recorded during growth in C+Y medium of the wild-type (wt), as well as the RT mutant strains each carrying a \textit{comA} mutation to prevent CSP export and allow measurement of basal-level expression of \textit{comCDE} (Fig. 2A). Calculation of RT to wt ratio (based on the mean of the four highest RLU values in each panel in Fig. 2A) revealed an 8.9- to 10.5-fold increase in basal-level expression of the \textit{comCDE} operon in the RT background.

![Fig. 2. Increased expression of \textit{comCDE} in non-competent RT cells. (A) \textit{comCDE} expression was monitored using a \textit{comC}::\textit{luc} transcriptional fusion (RLU, filled symbols) in \textit{comA}\textsuperscript{-} derivatives of the wt (R1568, black triangles and lines) and the RT strain (R1573, grey squares and lines) during growth (OD\textsubscript{492}, open symbols) in C+Y medium at 37 °C for 400 min. Cultures were inoculated at the following densities: 10\textsuperscript{7} (top panel), 2 x 10\textsuperscript{6} (central panel) and 4 x 10\textsuperscript{5} (bottom panel) cells ml\textsuperscript{-1}. Arrows indicate the four highest RLU values used for calculation of RT to wt ratios. (B) Detection of ComE by Western analysis with ComE antiserum (Methods). Lane 1, \textit{ΔcomCDE} mutant strain R315 (C); lanes 2 and 3, strain R800 (wt); lanes 4 and 5, strain R1564 (CEP\textsuperscript{comDE}); lanes 6 and 7, strain R1065 (RT). CSP and maltose were used at 100 ng ml\textsuperscript{-1} and 0.3 %, respectively.](Image)
Examination of the intracellular level of ComE by Western analysis confirmed that \textit{comCDE} expression was significantly increased in the RT mutant (Fig. 2B). The amount of ComE in RT cells was similar to that in CSP-induced wt cells (Fig. 2B, compare lanes 6 and 3). The results of transcriptional study and of Western analysis were thus fully consistent with the hypothesis that the RT mutation leads to transcriptional readthrough of \textit{comCDE}.

**The RT mutation confers a reduced-competence phenotype**

Preliminary experiments revealed that the RT mutation, despite increased \textit{comCDE} expression (see above), did not lead to competence derepression. To investigate the spontaneous competence development of the RT mutant in more detail, we took advantage of the previously constructed \textit{ssbB::luc} transcriptional fusion (Methods). Competence development of the wt strain R1556 and its RT derivative R1555 was monitored during growth in C+Y medium using different inoculum sizes. While with an inoculum of $10^7$ cells ml$^{-1}$ the peak of competence occurred after approximately 120 min incubation for the wt, spontaneous competence did not develop in the RT mutant culture (Fig. 3, top panel). With lower inocula, competence of the RT mutant was delayed by 60–70 min compared to the wt. In addition, competence was reduced two- to threefold, as judged from luciferase activity (Fig. 3, central and bottom panels). The latter effect might be a direct consequence of delayed competence, as competence is usually lower when it occurs at higher cell densities (Fig. 3, compare maximum RLU/OD values for the wt in each panel; and our unpublished observations).

The ability of RT (\textit{comA}$^-$) cells to respond to synthetic CSP was also compared to that of wt (\textit{comA}$^+$) cells in a mixed-culture experiment. Both strains responded equally to 50 ng CSP1 ml$^{-1}$ (Fig. 4). However, the yield of transformants for the RT mutant was reduced relative to that of the wt as CSP concentration was progressively diminished to 5 ng ml$^{-1}$, indicating that RT cells require more CSP than the wt for full competence. The extent of this relative reduction was 7-5- and 20-fold, respectively, with 10 and 5 ng CSP ml$^{-1}$ (Fig. 4). These data established that despite increased expression of the master competence-control operon \textit{comCDE}, the RT mutant displays reduced spontaneous competence and reduced response to CSP.

**Overexpression of \textit{comAB} suppresses the RT phenotype**

The data in Fig. 4 also indicated that the competence defect conferred by the RT phenotype could be bypassed by providing mutant cells with more CSP. At first sight, this observation seemed puzzling, since transcriptional readthrough of \textit{comCDE} should increase both the number of ComDE molecules and the amount of CSP made. However, this can be readily explained in light of previous evidence.
that shows that the export of CSP through ComAB is limiting in non-competent cells (Martin et al., 2000). RT cells could thus accumulate pre-CSP (intracellularly), and the excess of ComDE molecules over mature CSP would be responsible for the reduced-competence phenotype. This hypothesis was checked by introducing the ::spc190C-comA++ insertion into RT strain R848. Insertion of the spc cassette in front of comAB has previously been shown to lead to permanently enhanced expression of the operon (from the spc promoter) and to confer a CUP phenotype (Martin et al., 2000). The resulting strain, R854, displayed a CUP phenotype, as judged from its ability to transform at high levels when inoculated at 10^7 cells ml^{-1} in C+Y medium (Fig. 5, top panel). In contrast, the parental RT strain (R848) failed to transform, confirming the conclusion from the experiment in which competence was monitored with the ssbB::luc fusion (Fig. 3, top panel). With a lower inoculum (2 x 10^6 cells ml^{-1}), strain R854 displayed a typical CUP phenotype. It transformed throughout the culture (except when cells entered into the stationary phase of growth) (Fig. 5, bottom panel). In the same experiment, the RT strain achieved maximum transformation only after 165 min incubation, again in complete agreement with the experiment shown in Fig. 3 (central panel).

In addition, plating of strain R912 (an independently constructed strain combining the RT mutation and the ::spc190C-comA++ insertion; Table 1) on blood agar plates revealed that the overexpression of comAB also suppressed the ß-haemolytic phenotype typical of the RT mutant (data not shown; Guiral, 2004). Together, these data indicated that comAB overexpression is epistatic over the RT mutation, providing support to our hypothesis that the RT phenotype results, at least in part, from an imbalance between the intracellular concentration of ComDE and the amount of mature CSP produced.

**Ectopic expression of comDE mimics the RT phenotype**

It follows from the imbalance hypothesis that an increase in comDE expression in an otherwise wt background should mimic the RT phenotype. To test this, we constructed a strain, R1564 (CEPcomDE), which carries a second copy of the comDE genes placed at CEP to allow the regulated expression of both under the control of the maltose-inducible promoter P_M (Methods). As a control, strains R1564 and R1567, the latter of which carries an empty CEP (CEP0), were first grown in C+Y containing 0-3% sucrose, which minimizes expression from P_M. Both strains displayed spontaneous competence (Fig. 6A, top panel). However, competence was slightly delayed in the former.
ComDE basal levels affect competence

(... ~ 20 min) suggesting that residual transcription of comDE from PM might occur under these conditions, although no increase in the intracellular concentration of ComE compared to wt could be detected (Fig. 2, compare lanes 4 and 2). Strains R1564 and R1567 were then grown in C+Y containing 0.3% maltose. In contrast to strain R1567, strain R1564 did not develop spontaneous competence (Fig. 6A, bottom panel). Western analysis indicated that the intracellular concentration of ComE in R1564 cells grown under these conditions was slightly lower than that in non-competent RT cells (Fig. 2B, compare lanes 5 and 6) or in CSP-induced wt cells (Fig. 2B, compare lanes 5 and 3). We concluded from these observations that increased expression of comDE impedes competence development in S. pneumoniae.

Maltose-induced expression of comDE in strain R1564 not only prevented spontaneous competence but also conferred on this strain a β-haemolytic phenotype similar to that displayed by the RT mutant. R1564 cells plated on blood agar containing 0.2% glucose (or 0.2% maltose, data not shown) formed both spontaneous and CSP-induced β-haemolytic haloes (Fig. 6B). These data demonstrated that ectopic, PM-driven expression of comDE conferred a phenotype on strain R1564 indistinguishable from that of the RT mutant with respect to both competence defect and alolysis on blood agar plates.

DISCUSSION

Transcriptional readthrough from the tRNAArg gene upstream of comCDE increases basal-level expression of the comCDE operon and affects both spontaneous and CSP-induced competence, as demonstrated here with the RT mutant. That an increased expression of comCDE could result in competence inhibition was unexpected, since transcriptional readthrough of comCDE should increase in a balanced way both the number of ComDE molecules and the amount of (comC-encoded) pre-CSP made. This puzzling effect could be explained by taking into account a previous observation that CSP export capacity is limiting (Martin et al., 2000). In full agreement with this hypothesis, increased expression of the comAB genes, which encode the CSP exporter, was found to suppress the RT mutation. This finding suggested that the RT phenotype results, at least in part, from an imbalance between the intracellular concentration of ComDE and the amount of mature CSP produced.

These observations led to the prediction that an increase in basal-level expression of comDE only would also affect competence. This prediction was tested by placing a second copy of the comDE genes at an ectopic chromosomal location, under the control of the maltose-inducible promoter PM. Growth in the presence of a fully inducing concentration of maltose demonstrated that ectopic expression of comDE is sufficient to mimic the RT phenotype. At this stage we can only speculate on which component, ComD or...
ComE, is responsible for the reduced-competence phenotype and how this inhibitory effect is mediated. If excess ComD is responsible, the inhibitory effect could result directly from the mechanism of phosphorylation of the HK, as follows. Phosphorylation of the prototype of the HK superfamily, CheA, is known to involve an intersubunit mechanism (Surette et al., 1996). Assuming a similar phosphorylation mechanism for ComD, an increased concentration of ComD could impede the response to CSP by reducing the probability of interaction between two ligand-bound ComD monomers (i.e. CSP–ComD) required for autophosphorylation of ComD. With such a model, increasing CSP concentration (either through addition of synthetic CSP or by increasing CSP-export capacity) would suppress the inhibitory effect, as observed.

Alternatively, or in addition, excess ComE could be responsible for the inhibition of competence. In this model, the response regulator would have to act not only positively, to induce expression of the early com genes in response to circulating CSP, but also negatively, as a repressor of the comCDE operon and possibly of the comAB operon. This model would also fit the observations that the export of CSP is limiting in the RT mutant and that addition of synthetic CSP can bypass the reduced-competence phenotype of the mutant. Work is in progress to distinguish between the two models. Whatever the molecular explanation of the RT phenotype, our observation that an increase in comDE expression can prevent spontaneous competence induction illustrates that, contrary to current approaches, it might not be sufficient to overexpress a selected TCS to induce and characterize the regulon it controls.

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