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 tRNA^{Arg} 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.

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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 tRNA^{Arg} located immediately upstream of the *comCDE*

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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.

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).

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 reduced 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.

METHODS

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 (Bergé *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 μ g ml⁻¹, erythromycin (Ery) 0.1 μ g ml⁻¹, kanamycin (Kan) 500 μ g ml⁻¹, spectinomycin (Spc) 200 μ g ml⁻¹ or streptomycin (Sm) 400 μ g ml⁻¹, after phenotypic expression for 120 min at 37 °C.

Construction of CEP^{comDE}. 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 P_M, 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 *Nco*I/*Bam*HI, the *comDE* fragment (2086 bp) was ligated with *Nco*I/*Bam*HI-digested plasmid pCEP (Table 1). Transformation of strain R1556 with the ligation mixture and selection for Kan^R transformants was used to generate strain R1564 (CEP^{comDE}).

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, *ssbB* (Table 1). A direct correlation between luciferase activity and transformation level was observed under widely varying levels of competence (Bergé *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.

Strains	Genotype/description	Source/reference
<i>S. pneumoniae</i>		
R6	Non-capsulated D39 derivative	Laboratory stock
R800	R6 derivative	Martin <i>et al.</i> (1985)
R246	R800, but <i>hexAΔ3::ermAM</i> ; Ery ^R	Alloing <i>et al.</i> (1998)
R304	<i>nov1, rif23, str41</i> ; Nov ^R , Rif ^R , Sm ^R	Mortier-Barrière <i>et al.</i> (1998)
R315	R800, but <i>ΔcomCDE</i> ; Cm ^R	Alloing <i>et al.</i> (1998)
R391	R800, but <i>comA::kan</i> ; Kan ^R	Guiral <i>et al.</i> (2005)
R686	R800, but RT- <i>comC::lacZ</i> (pXF520), RT- <i>comC</i> ⁺ , <i>comA::ermAM</i> ^A , <i>ebg::spc</i> ; Cm ^R , Ery ^R , Spc ^R	Guiral <i>et al.</i> (2005)
R848	R246, but RT- <i>comC::lacZ</i> (pXF520), RT- <i>comC</i> ⁺ ; Cm ^R , Ery ^R	This study
R854	R848, but <i>::spc190^C-comA</i> ⁺ (by transformation with R467 chromosomal DNA; Martin <i>et al.</i> , 2000); Cm ^R , Ery ^R , Spc ^R	This study
R912	R800, but RT- <i>comC::lacZ</i> (pXF520), RT- <i>comC</i> ⁺ , <i>::spc190^C-comA</i> ⁺ ; Cm ^R , Spc ^R	This study
R1065	R800, but RT, <i>rpsL1</i> ; Sm ^R	Sung <i>et al.</i> (2001)
R1555	R800, but RT, <i>ssbB::luc</i> (pR424), <i>ssbB</i> ⁺ , <i>rpsL1</i> ; Cm ^R , Sm ^R	Guiral <i>et al.</i> (2005)
R1556	R800, but <i>ssbB::luc</i> (pR424), <i>ssbB</i> ⁺ , <i>rpsL1</i> ; Cm ^R , Sm ^R	This study
R1564	R800, but CEP ^{comDE} [by transformation with a ligation mixture of <i>NcoI/BamHI</i> -digested (pCEP and <i>comDE</i> PCR fragment); see Methods], <i>ssbB::luc</i> (pR424), <i>ssbB</i> ⁺ , <i>rpsL1</i> ; Kan ^R , Cm ^R , Sm ^R	This study
R1567	R800, but CEP ^o (by transformation with pCEP), <i>ssbB::luc</i> (pR424), <i>ssbB</i> ⁺ , <i>rpsL1</i> ; Kan ^R , Cm ^R , Sm ^R	Guiral <i>et al.</i> (2006b)
R1568	R800, but <i>comC::luc</i> (pR414; see Fig. 1, bottom), <i>comC</i> ⁺ , <i>comA::kan42</i> ^A , <i>rpsL1</i> ; Ery ^R , Kan ^R , Sm ^R	This study
R1573	R800, but RT- <i>comC::luc</i> (pR428; see Fig. 1, bottom), RT- <i>comC</i> ⁺ , <i>comA::kan42</i> ^A , <i>rpsL1</i> ; Cm ^R , Kan ^R , Sm ^R	This study
<i>Escherichia coli</i>		
LE392	F ⁻ e14 ⁻ (McrA ⁻) <i>hsdR514</i> (r _K ⁻ m _K ⁺) <i>glnV44 supF58 lacY1 galK2 galT22 metB1 trpR55</i>	Sambrook <i>et al.</i> (1989)
Plasmids		
pR414	ColE1 (p5.00) derivative; Ery ^R ; carries a <i>comC</i> -targeting fragment adjacent to <i>luc</i> ; insertion-duplication in <i>S. pneumoniae</i> generates a <i>comC::luc</i> (<i>comC</i> ⁺) fusion	Prudhomme & Claverys (2006)
pR424	ColE1 (pEVP3) derivative; Cm ^R ; carries an <i>ssbB</i> -targeting fragment adjacent to <i>luc</i> ; insertion-duplication in <i>S. pneumoniae</i> generates an <i>ssbB::luc</i> (<i>ssbB</i> ⁺) fusion	Chastanet <i>et al.</i> (2001)
pR428	pR424 derivative; Cm ^R ; carries a <i>comC</i> -targeting fragment, with the RT mutation, adjacent to <i>luc</i> ; insertion-duplication in <i>S. pneumoniae</i> generates a <i>comC::luc</i> (<i>comC</i> ⁺) fusion (Fig. 1, bottom)	Guiral <i>et al.</i> (2005)
pCEP	pSC101 derivative; Spc ^R , Kan ^R ; carries the <i>S. pneumoniae</i> chromosomal expression platform, CEP	Guiral <i>et al.</i> (2006b)
Primers		
	Sequence* ; gene ; position [†]	
comDnco	GAAAGAGccATGGATTTATTTGGATTTGG; <i>comD</i> ; +138	
BM93	GccggaTcTAATTGTCAATCACTTTTGAG; <i>comE</i> ; +2240	

*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 LucyI luminometer (Anthos). The use of clear-bottomed plates allowed the measurement of OD₄₉₂ 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^{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 (8×10^6) were inoculated into 4 ml medium and incubated at 37 °C for ~3 h (up to OD₅₅₀ 0.15). For maltose induction, 0.3% (w/v) maltose was substituted for sucrose. For CSP induction, CSP1 (100 ng ml⁻¹) was added, and cells were incubated for 20 min (final OD₅₅₀ 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 × 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 $\sim 2 \times 10^8$ 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 *comCDE*

The hypothesis that the RT mutation destabilizes the putative transcription terminator of tRNA^{Arg} (Fig. 1, top) and leads to transcriptional readthrough of *comCDE* (Martin *et al.*, 2000) was checked using a *comC::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 *comA*⁻ mutation to prevent CSP export and allow measurement of basal-level expression of *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 *comCDE* operon in the RT background.

Fig. 2. Increased expression of *comCDE* in non-competent RT cells. (A) *comCDE* expression was monitored using a *comC::luc* transcriptional fusion (RLU, filled symbols) in *comA*⁻ derivatives of the wt (R1568, black triangles and lines) and the RT strain (R1573, grey squares and lines) during growth (OD₄₉₂, open symbols) in C+Y medium at 37 °C for 400 min. Cultures were inoculated at the following densities: 10^7 (top panel), 2×10^6 (central panel) and 4×10^5 (bottom panel) cells ml⁻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, Δ *comCDE* mutant strain R315 (C); lanes 2 and 3, strain R800 (wt); lanes 4 and 5, strain R1564 (CEP^{comDE}); lanes 6 and 7, strain R1065 (RT). CSP and maltose were used at 100 ng ml⁻¹ and 0.3%, respectively.

Fig. 3. The RT strain displays reduced spontaneous competence. Luciferase expression from an *ssbB::luc* transcriptional fusion, which reports competence (RLU/OD; filled symbols), and OD₄₉₂ (open symbols) were recorded for 300 min during incubation in C+Y medium at 37 °C (Methods) for the wt strain (R1556, black triangles and lines) and its RT derivative (R1555, grey squares and lines). Cultures were inoculated at the following densities: 10^7 (top panel), 2×10^6 (central panel) and 4×10^5 (bottom panel) cells ml⁻¹.

Examination of the intracellular level of ComE by Western analysis confirmed that *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 *comCDE*.

The RT mutation confers a reduced-competence phenotype

Preliminary experiments revealed that the RT mutation, despite increased *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 *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 (*comA*⁻) cells to respond to synthetic CSP was also compared to that of wt (*comA*⁻) cells in a mixed-culture experiment. Both strains responded equally to 50 ng CSP 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 *comCDE*, the RT mutant displays reduced spontaneous competence and reduced response to CSP.

Overexpression of *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 *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

Fig. 4. The RT mutation reduces response to CSP. Cultures of strains R686 (RT, *comA*⁻) and R391 (*comA*⁻) grown in CAT were mixed at a ratio of 1:1 (OD₅₅₀ 0.1) and activated for 10 min at 37 °C using CSP concentrations ranging from 5 to 50 ng ml⁻¹, then transformed to Sm^R (Methods). RT and wt transformants were scored respectively as Sm^R-Spc^R (grey bars) and Sm^R-Kan^R (white bars) (Methods). The fold-reduction in transformation of the RT strain relative to the wt (calculated as the ratio of wt to RT transformants) with decreasing CSP concentrations is indicated (▲).

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 *::spc190^C-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 10⁷ 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 × 10⁶ 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 *::spc190^C-comA*⁺ insertion; Table 1) on blood agar plates revealed that the overexpression of *comAB* also

Fig. 5. Suppression of the competence-down phenotype of the RT mutant by overexpression of *comAB*. Growth (OD₅₅₀, open symbols) of cultures in C+Y medium was monitored and spontaneous transformability (expressed as percentage of transformants, filled symbols) was measured every 15 min (from 90 to 210 min) by transforming aliquots to Sm^R (Methods), as previously described (Alloing *et al.*, 1996). Cultures were inoculated at 10⁷ (top panel) and 2 × 10⁶ (bottom panel) cells ml⁻¹, respectively. Strain R848 (RT), grey squares and lines; strain R854 (RT and *::spc190^C-comA*⁺), black triangles and lines.

suppressed the β -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 (CEP^{comDE}), 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

Fig. 6. Increased expression of *comDE* at CEP mimics the phenotype of the RT strain. (A) Growth (OD₄₉₂, open symbols) and development of competence (RLU/OD, filled symbols) of a wt strain (R1567, black triangles and lines), which carries an empty CEP (CEP⁰), and its CEP^{comDE} derivative (R1564, grey squares and lines) were monitored as described in the legend to Fig. 3. Cultures were inoculated at 2×10^6 cells ml⁻¹ in C+Y medium containing 0.3% sucrose (C-suc, top panel) or 0.3% maltose (C-mal, bottom panel). (B) Detection of β-haemolytic haloes in 5.5% horse blood CAT agar plates containing 0.2% glucose. After overnight incubation at 37 °C, the RT strain R1555 and the CEP^{comDE} strain R1564 displayed spontaneous β-haemolytic haloes. Spots of CSP (1 μl, 100 ng, indicated by white pointers) deposited immediately after plating induced the formation of β-haemolytic haloes in both strains. The 1× concentration corresponds to 2.5×10^6 cells per plate. Similar data were obtained in medium containing 0.2% maltose, while β-haemolytic haloes were abolished for strain R1564 in medium containing 0.2% sucrose (data not shown; Guiral, 2004).

(~20 min) suggesting that residual transcription of *comDE* from P_M 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, P_M-driven expression of *comDE* conferred a phenotype on strain R1564 indistinguishable from that of the RT mutant with respect to both competence defect and allolysis on blood agar plates.

DISCUSSION

Transcriptional readthrough from the tRNA^{Arg} 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 P_M. 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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