Construction and evaluation of a chromosomal expression platform (CEP) for ectopic, maltose-driven gene expression in *Streptococcus pneumoniae*

Sébastien Guiral, Vincent Hénard, Maria-Halima Laaberki,[†]
Chantal Granadel, Marc Prudhomme, Bernard Martin
and Jean-Pierre Claverys

Correspondence
Jean-Pierre Claverys
claverys@ibcg.biotoul.fr

Laboratoire de Microbiologie et Génétique Moléculaires, UMR 5100 CNRS-Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse Cedex 9, France

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In this paper, the construction and evaluation of a chromosomal expression platform (CEP), which allows controlled gene expression following ectopic integration into the chromosome of *Streptococcus pneumoniae*, is described. CEP is based on the well-studied maltosaccharide-inducible system. To facilitate integration at CEP, a plasmid, pCEP, capable of replication in *Escherichia coli*, but not in *S. pneumoniae*, was assembled. This plasmid contains an expression/selection cassette flanked on each side by more than 2 kb of pneumococcal DNA. The cassette comprises a maltose-inducible promoter, P_M , separated from a kanamycin-resistance gene by *Nco*I and *Bam*HI cloning sites. Clones harbouring the gene of interest integrated at CEP under the control of P_M can be obtained through direct transformation of an *S. pneumoniae* recipient with ligation products between that gene and *Nco*I/*Bam*HI-digested pCEP DNA, followed by selection for kanamycin-resistant transformants.

INTRODUCTION

Streptococcus pneumoniae is widely used as a model Gram-positive pathogenic bacterium with low GC content, particularly since natural genetic transformation constitutes a convenient tool for molecular genetic studies of virulence. The involvement of many *S. pneumoniae* genes in virulence has been investigated through gene inactivation. An important step to validate inactivation data involves complementation of the inactivated function. While the *amyE* ectopic chromosomal platform is in routine use for complementation studies in *Bacillus subtilis* (Kong *et al.*, 1993), no equivalent system has been developed so far for *S. pneumoniae*. Complementation has therefore been achieved in several different ways. The complementing gene can be cloned into a replicative plasmid and introduced by transformation (Balganesh & Lacks, 1985; Prats *et al.*, 1985). However, multicopy complementation can lead to perturbations of cell physiology for various reasons, including the presence of the replicative plasmid itself. Thus, a recombinant plasmid

carrying the *nisRK* two-component regulatory system and the gene of interest cloned under the control of the nisin-inducible *nisA* promoter has been reported to confer sensitivity to temperature (Luo *et al.*, 2003).

To avoid copy-number effects, alternative strategies based on single-copy gene expression have therefore been developed over the years. One of these makes use of the ectopic integration of a recombinant plasmid (non-replicative in *S. pneumoniae*) in the chromosome through transformation (Vasseghi *et al.*, 1981; Mannarelli & Lacks, 1984). A chromosome-targeting fragment is inserted into the recombinant plasmid to obtain ectopic integration. A 354 bp fragment from the *ami* operon of *S. pneumoniae* has thus been used to direct integration of a recombinant plasmid harbouring the firefly luciferase gene, *luc*, and luciferase activity is observed in pneumococcal cells (Stieger *et al.*, 1999). Similarly, a *qsrA* fragment has been inserted into a new non-replicative plasmid, pEVP3qsrI, to obtain ectopic expression of the *S. pneumoniae cbpD* gene at the *qsrA* locus (Kausmally *et al.*, 2005). In this example, the recombinant plasmid carries two regions of homology with the recipient chromosome, *qsrA* (886 bp) and *cbpD* (1377 bp), and can therefore integrate preferentially at *cbpD*. Integration at *qsrA* is obtained through the use of a recipient deleted for *cbpD*. It can thus be necessary for efficient ectopic integration to delete from the recipient chromosome the

[†]Present address: UMR1225 Interactions Hôtes-Agents Pathogènes, INRA-Ecole Nationale Vétérinaire de Toulouse, 23 chemin des Capelles BP 87614, 31076 Toulouse Cedex 3, France.

Abbreviations: CSP, competence-stimulating peptide; CEP, chromosomal expression platform; Ery, erythromycin; Kan, kanamycin; RLU, relative luminescence unit; Sm, streptomycin.

counterpart of the complementing gene. In any case, duplication of the chromosome-targeting fragment occurs during integration of the recombinant plasmid and creates the potential drawbacks that the plasmid is either amplified (hence is no longer in single copy) or is excised and lost (Vasseghi & Claverys, 1983).

A better system should thus allow stable single-copy integration at a neutral chromosomal site. Integration of an ectopic gene copy at the *bgaA* locus might fulfil this criterion (Robertson *et al.*, 2002, 2003; Chan *et al.*, 2003). However, although it has been reported that insertion mutations of various kinds into *bgaA* do not affect growth, the function(s) of BgaA is not fully understood. Taking into account the overall length of the gene (~ 6.7 kb) and the surface location of the protein, it is not unlikely that BgaA has other function(s) in addition to the previously documented β -galactosidase activity (Zähner & Hakenbeck, 2000). It is therefore difficult to consider *bgaA* as a neutral chromosomal site. Ectopic expression at the *malM* locus, which encodes amylomaltase (Stassi *et al.*, 1982), has been used to demonstrate that the 5' to 3' exonuclease activity of DNA polymerase I is essential for *S. pneumoniae* (Díaz *et al.*, 1992), but this system is not designed to allow easy further use. Ectopic expression of a gene inserted between *aga*, the gene encoding α -galactosidase, and *rafE*, under the control of a raffinose-inducible promoter, has also been used (Luo *et al.*, 2003). This insertion site is probably neutral with respect to the physiology of recipient cells, but this system also is not designed for easy repeated use. In view of these limitations, the construction of a new convenient single-copy gene-expression system, which is still lacking for *S. pneumoniae*, would be useful. In this paper, we describe the construction and evaluation of a versatile chromosomal expression platform (CEP), pre-assembled on a plasmid (pCEP) capable of replication in *Escherichia coli*, but not in *S. pneumoniae*. pCEP allows placement of the gene of interest under the control of a maltosaccharide-inducible promoter and provides flanking homology for its integration into a region of the pneumococcal chromosome originally devoid of expressed sequences.

METHODS

Bacterial strains, growth conditions and transformation. *S. pneumoniae* strains and plasmids used in this study are described in Table 1. Precultures were grown at 37 °C in CAT or in C+Y medium (Bergé *et al.*, 2002) to OD₅₅₀ 0.4, then centrifuged, resuspended in fresh C+Y medium containing 15% (v/v) glycerol and kept frozen at -70 °C. These precultures were used to initiate cultures in C+Y at 2×10^6 cells ml⁻¹. Transformation was performed as described previously (Martin *et al.*, 2000). Transformants were selected by plating in 10 ml CAT agar supplemented with 4% horse blood, followed by challenge with a 10 ml overlay containing 0.1 µg ml⁻¹ erythromycin (Ery), 500 µg ml⁻¹ kanamycin (Kan) or 400 µg ml⁻¹ streptomycin (Sm), after phenotypic expression for 120 min at 37 °C.

Monitoring of luciferase activity and growth. Luciferase activity was monitored in living pneumococcal cells as previously described (Prudhomme & Claverys, 2006). Luminescence, which reports luciferase

activity (expressed in relative luminescence units, RLU), was measured in cultures grown in a Corning NBS 96-well (320 µl volume) white plate with clear bottom incubated at 37 °C for up to 6 h in a LucyI luminometer (Anthos). The use of a clear-bottomed plate allowed the monitoring of OD₄₉₂ in parallel with luminescence.

RESULTS AND DISCUSSION

Choice of an inducible system for controlled gene expression at CEP

The regulated systems previously used for controlled gene expression in *S. pneumoniae* depend on sugars, antibiotics, and antibacterial or regulatory peptides for induction. Maltose- (Nieto *et al.*, 2000), raffinose- (Luo *et al.*, 2003) or fucose-regulated promoters (Chan *et al.*, 2003) have thus been used, as well as tetracycline- (Stieger *et al.*, 1999), nisin- (Luo *et al.*, 2003) or competence-stimulating peptide (CSP)1/bacteriocin inducing peptide (BIP)1-inducible promoters (Kausmally *et al.*, 2005).

In choosing a regulatory system for inclusion at CEP, we were reluctant to use sublethal concentrations of antibiotic (tetracycline) or antibacterial peptide (nisin) as inducer. We also ruled out the use of CSP1, which induces the competence regulon (Dagkessamanskaia *et al.*, 2004; Peterson *et al.*, 2004), or BIP1, which induces the Blp regulon (de Saizieu *et al.*, 2000), because they could not be considered to be neutral with respect to pneumococcal physiology. We therefore retained a sugar-inducible system and selected the maltosaccharide-inducible system because of the extensive studies conducted by Lacks, Espinosa and coworkers. P_M, the inducible promoter of the *malMP* operon (Stassi *et al.*, 1982), has previously been used for the construction of a tightly regulated plasmid vector for *S. pneumoniae* (Nieto *et al.*, 2000). P_M is regulated by the MalR repressor, the binding of MalR to P_M being relieved when pneumococcal cells are grown in maltose-containing media (Lacks, 1968; Nieto *et al.*, 1997). A replicative plasmid containing the *malR* repressor gene (pAPM22; Puyet *et al.*, 1993; Table 1) is available to increase the intracellular amount of MalR, thus ensuring high levels of repression.

Choice of CEP location

In our choice of a chromosomal location for CEP we looked for a transcriptionally silent site, at which integration of exogenous material would not perturb any known cellular function. We therefore chose to introduce CEP immediately downstream of the well-studied *ami* operon (Alloing *et al.*, 1990), and designed it so that its integration would occur by replacement-recombination. Upon integration, CEP substitutes for a truncated IS1167 element (Fig. 1) between nucleotides 1 671 049 and 1 672 299 in the 2 038 615 bp R6 genome (Hoskins *et al.*, 2001). When an empty CEP is integrated, DNA segments of similar size are exchanged. Two transcription terminators, which flank CEP on each side, are retained after integration (Fig. 1).

Table 1. Strains, plasmids and primers used in this study

Ap, ampicillin; Cm, chloramphenicol; Nov, novobiocin; Rif, rifampicin; Spc, spectinomycin.

Strains	Genotype/description	Source/reference
<i>S. pneumoniae</i>		
R6	Non-capsulated D39 derivative	Laboratory stock
R304	<i>nov1</i> , <i>rif23</i> , <i>str41</i> ; Nov ^R , Rif ^R , Sm ^R	Mortier-Barrière <i>et al.</i> (1998)
R800	R6 derivative	Martin <i>et al.</i> (1985)
R1474	R800, but CEP ^{luc} [by transformation with a ligation mixture of <i>NcoI</i> / <i>Bam</i> HI-digested (pCEP and <i>luc</i> PCR fragment); Fig. 1]; Kan ^R	This study
R1488	R1474, but carries plasmid pAPM22; Kan ^R , Ery ^R	This study
R1567	R800, but CEP (by transformation with pCEP; Fig. 1), <i>ssbB::luc</i> (pR424), <i>ssbB</i> ⁺ , <i>rpsL1</i> ; Kan ^R , Cm ^R , Sm ^R	This study
<i>E. 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		
pAPM22	pLS1 derivative containing the <i>malR</i> gene cloned under the control of the <i>tet</i> gene promoter; Ery ^R	Puyet <i>et al.</i> (1993)
pCEP	pJPB209 derivative carrying CEP (Fig. 1); Spc ^R , Kan ^R	This study
pJPB209	pSC101 derivative carrying the <i>spc</i> gene; Spc ^R	Pichoff <i>et al.</i> (1995)
pR410	pEMcat derivative carrying the <i>kan</i> gene; Ap ^R , Kan ^R	Sung <i>et al.</i> (2001)
pR414	ColE1 (p5.00) derivative carrying the <i>luc</i> gene; Ery ^R	Prudhomme & Claverys (2006)
Primers		
	Sequence*[†]; gene; position[†]	
BM105	ATTCGCaagcTTTCCCTTGAAGCTAGTCGAAG; internal to <i>amiE</i> ; +4559	
BM106	TCAAACCTTATCAAGCGCAATGCC; internal to <i>IS1167</i> ; +6675	
BM107M	GGCATTGCGCTTGATAAGTTTGATATAAAATTCAAGCTCCCTAAGGAGG ; upstream of <i>malM</i> ; −245	
BM108M	GGATCCCCAGCTTGCGCGcCATggTAGCACCTCGTGTGTAA ; upstream of <i>malM</i> ; +3	
BM109	CGCGCAAGCTGGGGATCC; upstream of <i>aphIII</i> (<i>kan</i>); −85	
BM110.1	GTagAgATCTAGGTACTAAAACAATTTCATCCAGTAA; downstream of <i>aphIII</i> (<i>kan</i>); +804	
BM111	GTTTTAGTACCTAGATCTCTACACAAAAATAGGCTCCATAATATC ; upstream of <i>IS1167</i> ; +7925	
BM112	CCTGCcGGGCTGAACCAAGTCATCCCAGCAACAAATGG; internal to <i>treP</i> ; +1250	
MP162	GCTGAAGGAGGAaccATGgGATCCGCC; upstream of <i>luc</i> ; −15	
MP163	GGTACCCGgaGATCTTACAATTGGGC; downstream of <i>luc</i> ; +1667	

*Sequences shown in bold in BM107M, BM108M and BM111 are complementary to BM106, BM109 and BM110.1, respectively. Sequences underlined correspond to *Bam*HI (BM108M and BM109), *NcoI* (BM108M and comDnc), *Hind*III (BM105), *Xma*I (BM112) and *Bgl*II (BM111 and BM110.1) restriction sites. Small letters in the oligonucleotide sequence indicate nucleotide changes to introduce convenient restriction sites.

[†]With respect to the ATG of the corresponding gene (or of the first gene of the operon); − and + indicate, respectively, upstream and downstream.

Design of CEP

The ease of introduction into the pneumococcal chromosome and the ease of repeated use of CEP were the two most important criteria defined for CEP. This led us to construct pCEP, a recombinant plasmid harbouring an expression/selection cassette and capable of replication in *E. coli* but not in *S. pneumoniae*. The cassette contains the maltose-inducible promoter P_M adjacent to the *aphIII* gene (called hereafter *kan*), which confers resistance to Kan for selection of single-copy transformants in *S. pneumoniae*.

NcoI and *Bam*HI restriction sites located between P_M and the *kan* gene are available to insert downstream of P_M any DNA fragment flanked by appropriate restriction sites, through ligation to *NcoI*/*Bam*HI-digested pCEP DNA. The *NcoI* site can also be used to fuse the gene under study directly to the ATG of *malM* (Fig. 1), thereby removing the potential regulatory signals of the cloned gene and rendering its expression totally dependent on P_M.

The cassette is flanked on each side by more than 2 kb of pneumococcal DNA from the chromosomal region

Fig. 1. Organization of the chromosomal expression platform, CEP. (A) Primers used to construct pCEP and to generate CEP^{luc} (Table 1, and Results and Discussion) are shown with horizontal arrows indicating primer orientation. Dotted lines (bordered by BM106 and BM111) indicate the *IS1167* remnant sequences deleted and replaced by the P_M -*kan* segment. The *amiE* and *amiF* genes belong to the oligopeptide permease *ami* operon (Alloing *et al.*, 1990). *treR* and *treP* are part of a predicted trehalose utilization system. *ter*, transcription terminator; P_M , maltose-inducible promoter; N and B, *NcoI* and *BamHI* restriction sites, respectively. (B) Sequence of the P_M DNA fragment. The previously identified (Nieto *et al.*, 1997) -35 and -10 regions are underlined and the MalR binding site is boxed. The residues flanking the ATG of *malM* (also boxed) have been modified in primer BM108M to introduce an *NcoI* site (Table 1). Note that our control sequencing revealed that the sequence of the P_M fragment differed from that initially published by Stassi *et al.* (1982) at several positions (due to underestimations in the number of residues at runs of A or T). It also differs by a block of 10 residues (underlined italics) apparently mislocated (vertical arrow) in the sequence shown in Fig. 2 in Nieto *et al.* (1997). However, our sequence is 100% identical to the sequence reported for the R6 strain (Hoskins *et al.*, 2001). Note also that pCEP plasmid DNA generates no transformants resistant to methotrexate (a phenotype conferred by *ami* mutations) in a wild-type recipient strain, which strongly suggests that pCEP carries no mutation in the *ami* region. The *tre* region has not been sequenced.

downstream of the *ami* operon (Fig. 1), a situation predicted to strongly favour cassette insertion over integration of the entire pCEP plasmid during transformation in *S. pneumoniae* (Pozzi & Guild, 1985). Transformants harbouring the appropriate construct can therefore be isolated directly following transformation of an *S. pneumoniae* strain with ligation products, with no need for an intermediate cloning step in *E. coli*. There are two main advantages to this strategy. First, the efficiency of pneumococcal transformation is much higher than that of *E. coli*. Second, it avoids potential cloning problems that arise in *E. coli* because of the previously documented frequent toxicity of cloned pneumococcal DNA fragments (Martin *et al.*, 1989).

Construction of pCEP and integration of CEP into the pneumococcal chromosome

Plasmid pCEP carries a chromosomal fragment, bordered by primers BM105 and BM112, in which most of the *IS1167*

remnant sequences (1249 bp) are substituted by a 1140 bp segment containing P_M and the *kan* gene (Fig. 1). Construction of the engineered BM105–BM112 segment involved several PCR reactions with the AccuTaq LA DNA polymerase (Sigma), as follows (see primers with arrows in Fig. 1). The P_M and the *Kan*^R fragments were generated with primer pairs BM107M/BM108M and BM109/BM110.1 (Table 1) using R800 chromosomal DNA and plasmid pR410 DNA as template, respectively (Fig. 1). Fragments corresponding to the leftward and rightward flanking chromosomal regions were amplified from R800 chromosomal DNA with BM105/BM106 and BM111/BM112 primer pairs (Table 1), respectively (Fig. 1). After purification with a QIAquick nucleotide removal kit (Qiagen), fragments BM105–BM106 and BM107M–BM108M in one reaction, and BM109–BM110.1 and BM111–BM112 in another reaction, were mixed and used as template to generate, respectively, the BM105–BM108M and BM109–BM112 fragments (Fig. 1). The BM105–BM112 final PCR product was then

amplified using the latter two PCR fragments as template. This fragment was digested with *HindIII/XmaI* and cloned into *HindIII/XmaI*-digested *E. coli* plasmid pJPB209 (Table 1) to generate plasmid pCEP.

Integration of CEP was readily obtained by transformation of the wild-type strain R800 with plasmid pCEP DNA and selection for Kan^R transformants. The frequency of Kan^R transformants was similar to that of Sm^R transformants obtained with the chromosomal marker *str41* (data not shown). Since the Kan^R cassette is flanked on each side by more than 2 kb of pneumococcal DNA, integration of the engineered P_M-*kan* segment in place of the IS1167 remnant was expected to occur at a much higher frequency than insertion of the entire pCEP molecule through insertion-duplication mechanisms (Méjean *et al.*, 1981; Pozzi & Guild, 1985). Analysis of a few Kan^R transformants confirmed the absence of pCEP vector sequences (data not shown). One of these transformants retained for further analysis, strain R1567, displayed growth and transformation properties indistinguishable from those of the parental strain (data not shown, but see Guiral *et al.*, 2006), suggesting that modifications introduced downstream of the *ami* operon have no significant effect on pneumococcal physiology.

Insertion of the *luc* gene at CEP

To evaluate ectopic gene expression at CEP, we used the *luc* gene as transcriptional reporter. Its placement at CEP under the control of P_M was achieved as follows. A DNA fragment containing *luc* was amplified with the MP162/MP163 primer pair using plasmid pR414 DNA as template (Table 1). After digestion with *NcoI* and *BamHI*, the fragment (1657 bp) was ligated with *NcoI/BamHI*-digested plasmid pCEP, and strain R800 was transformed with the ligation mixture. Ten out of 10 Kan^R transformants analysed harboured the CEP^{*luc*} construct (Fig. 1). One of them was retained as strain R1474. Note that the sequence of the *luc* gene was not checked, but further experiments revealed that it encoded a functional luciferase (see below).

Evaluation of ectopic, P_M-driven *luc* expression

The effect of sugar concentrations on growth of pneumococcal cells was first evaluated (Fig. 2). In C+Y containing only 0.1% sucrose, the growth rate was essentially unaffected, but cells stopped growing at an OD₄₉₂ value of 0.4, indicating that sucrose was limiting (Fig. 2A, C). Addition of 0.05 and 0.1% maltose increased final OD values, indicating that maltose was metabolized and suggesting that its concentration declined progressively with growth. Cultures with 0.2 and 0.4% maltose attained final OD values (~0.8) similar to those of cultures in the presence of 0.3% sucrose (Fig. 2, compare panels A and C with panels B and D). In C+Y containing 0.3% sucrose, addition of 0.05–0.4% maltose had no significant effect on growth rate and final OD values, indicating that sucrose was not limiting (Fig. 2B, D). Similar data were obtained when glucose (0.1 and

Fig. 2. Effect of sugar concentrations on growth of pneumococcal cells and on *luc* expression. Strains R1474 (CEP^{*luc*}; panels A and B) and R1488 (CEP^{*luc*} and the cloned *malR* gene, panels C and D) were grown in C+Y medium containing 0.1% (panels A and C) or 0.3% (panels B and D) sucrose (suc, open circles), or sucrose plus the following concentrations of maltose (filled symbols): 0.05% (triangles), 0.1% (squares), 0.2% (diamonds) or 0.4% (circles). Maltose was added at 60 min (+mal, black arrows). OD₄₉₂ (grey lines) and luciferase activity (expressed in RLU, black lines in panels B and D) were recorded during incubation at 37 °C for 300 min.

0.3%) was substituted for sucrose (data not shown). A concentration of 0.3% sucrose was employed in further expression studies.

Expression of the *luc* gene was then monitored in cultures of strain R1474 (CEP^{*luc*}) grown in C+Y with 0.3% sucrose and various concentrations of maltose (Fig. 2B). Luciferase activity increased from 8.2 ± 0.4 RLU in the control culture without maltose to 54.3 ± 1.6 RLU in the presence of maltose, independent of the concentration between 0.05 and 0.4% (Fig. 2B). Induction of *luc* expression in the presence of maltose was thus significant. That maltose induction could readily occur in the presence of sucrose was expected from previous studies (Lacks, 1968). The extent of

induction (6.6 ± 0.2 -fold increase in luciferase activity) was similar to that observed for amylomaltase levels in wild-type strains grown in the presence of sucrose and maltose versus sucrose only (6.8 -fold increase) (Lacks, 1968).

To investigate whether an increase in the intracellular level of the MalR repressor would lead to a better repression of the P_M promoter, *luc* expression was monitored in a derivative of strain R1474 containing a recombinant plasmid carrying the *malR* gene (strain R1488; Table 1). Luciferase activity in the control culture without maltose was reduced to 3.2 ± 0.1 RLU (Fig. 2D), suggesting that repression of P_M was slightly reinforced by an increase in the intracellular level of MalR. In the presence of maltose (within the range 0.05 – 0.4 %), luciferase activity increased to 37.8 ± 1.2 RLU (10.8 ± 0.4 -fold increase compared to the control culture; Fig. 2D).

Expression of the *luc* gene was then measured in cultures grown in the presence of concentrations of maltose below 0.05 % (Fig. 3). Luciferase activity was close to the maximum in the presence of 0.008 % maltose (48.9 ± 0.6 compared to 53.9 ± 0.2 RLU with 0.04 % maltose). While no significant induction of *luc* could be detected with 0.000064 % ($0.64 \mu\text{g ml}^{-1}$) maltose, intermediate expression values were obtained with 0.00032 % ($3.2 \mu\text{g ml}^{-1}$)

Fig. 3. Measurement of P_M -driven expression of *luc* at CEP. Strain R1474 was grown in C+Y medium containing 0.3 % sucrose (suc, open circles) or 0.3 % sucrose plus 0.000064 % ($0.64 \mu\text{g ml}^{-1}$; open squares), 0.00032 % ($3.2 \mu\text{g ml}^{-1}$; open triangles), 0.0016 % (open diamonds), 0.008 % (filled squares) or 0.04 % (filled triangles) maltose. Maltose was added at 65 min (+mal, black arrow). Luciferase activity (black lines, expressed in RLU) and OD_{492} (grey lines) were recorded during incubation at 37 °C for 300 min. Note that maximum luciferase yields (grey arrows) occurred at higher OD values (indicated by horizontal lines) with increasing maltose concentration ($OD_{492} \sim 0.165$, 0.275 and 0.415 with 0.00032 , 0.0016 and 0.008 – 0.04 % maltose, respectively), which suggests that maltose could be used for growth even in the presence of non-limiting sucrose.

and 0.0016 % maltose (17.5 ± 0.4 and 38.5 ± 1.3 RLU, respectively), indicating that maltose concentration can be adjusted to fine-tune gene expression at CEP.

Concluding remarks

CEP has recently been used to allow P_M -driven expression of a second copy of the *comDE* genes which control competence development in *S. pneumoniae* (Guiral *et al.*, 2006). The CEP^{comDE} construct has proved decisive in the demonstration that increased basal-level expression of the ComDE two-component regulatory system impedes competence development in *S. pneumoniae* (Guiral *et al.*, 2006).

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