Engineering of quorum-sensing systems for improved production of alkaline protease by *Bacillus subtilis*

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ABSTRACT


Aim: Engineering of Rap-Phr quorum-sensing systems of *Bacillus subtilis* and subsequent evaluation of the transcription of the *aprE* gene, encoding a major extracellular alkaline protease.

Methods and Results: Addition of synthetic Phr pentapeptides to the growth medium, or overproduction of pre-Phr peptides, slightly improved the transcription of the *aprE* gene in *B. subtilis*. Disruption of certain *rap* genes similarly improved the transcription of the *aprE* gene. The production of extracellular proteolytic enzymes was increased when the *rapA* mutation was combined with a *degU32* (Hy) mutation for hyper-secretion.

Conclusions: Certain Rap-Phr systems of *B. subtilis* seem to suppress extracellular AprE production. Although this may be an important feature under natural conditions, repression of AprE production by these systems is not desirable under fermentation conditions.

Significance and Impact of the Study: Although the levels of *aprE* transcriptional increase in this study are moderate, engineering of Rap-Phr systems may be used to improve the yield of *Bacillus* strains that are used for the production of the extracellular protease AprE, or *Bacillus* strains that use of the *aprE* promoter for the production of a heterologous protein.

Keywords: AprE, *Bacillus subtilis*, gene regulation, protein secretion, quorum-sensing, Rap-Phr.

INTRODUCTION

*Bacillus subtilis* and related bacilli have the capacity to secrete a variety of proteins into their environment, at frequently high concentrations (Tjalsma et al. 2000; Antelmann et al. 2001). This has led to the commercial exploitation of bacilli as ‘cell factories’ for secreted enzymes. Regulation of the synthesis of secretory proteins, especially one of the major extracellular proteases, AprE, has been studied in detail for more than 10 years (see Ferrari et al. 1988; Dahl et al. 1992; Jan et al. 2000). In soil, the natural habitats of *B. subtilis*, secreted proteases are frequently synthesized as part of an adaptive response to changes in the environment, allowing the cell to benefit optimally from the available resources. To respond to these changes, *B. subtilis* makes use of complex signal transduction systems to sense a wide variety of extracellular stimuli. For survival in a complex environment, two-component regulatory systems, each consisting of a sensor kinase and a response regulator, are necessary. Known two-component systems are involved in various processes, such as competence development (Dubnau 1991), protein secretion (Kunst et al. 1994; Darmon et al. 2002), synthesis of peptide antibiotics and bacteriocins (Marahiel et al. 1993; Stein et al. 2002) and sporulation (Grossman 1995). Upon phosphorylation of a certain response regulator...
by its cognate sensor kinase, the transcription of specific genes is induced. The fine-tuning of these regulatory systems can be governed by intracellular response regulators aspartyl phosphatases (Raps), and their antagonistic phosphatase regulators (Phrs). The Raps can dephosphorylate response regulators, thereby temporally ‘overruling’ the action of sensor kinases (Gray 1997). The Phr peptides serve as cell density-signaling molecules and inhibit the Rap phosphatases (Koetje et al. 1997). While the Rap phosphatases remain in the cytoplasm, Phr peptides contain an amino-terminal signal peptide and are exported as pro-peptides, most likely via the permease (Opp) system, Phr peptides specifically inhibit the re-import by cells in the culture, via the oligopeptide transport system, Rap peptides, with a weakly conserved X sequence. After re-import by cells in the culture, via the oligopeptide permease (Opp) system, Phr peptides specifically inhibit the activity of their cognate Rap phosphatase (Solomon et al. 1996; Perego 1997, 1998). However, four of the 11 known rap genes are not followed by functional phr genes, suggesting that these are not involved in quorum-sensing processes. Nevertheless, it cannot be excluded that these four Raps respond to Phr peptides encoded in other operons.

Interestingly, one of the rap-phr operons found on an endogenous rolling-circle plasmid from B. subtilis (Meijer et al. 1998), plays an indirect role in protein secretion enzymes in Rap60 represses the production and secretion of proteolytic (Koetje et al. 1997). Further extracellular processing results in active Phr penta-peptides, with a weakly conserved X sequence. After re-import by cells in the culture, via the oligopeptide permease (Opp) system, Phr peptides specifically inhibit the activity of their cognate Rap phosphatase (Solomon et al. 1996; Perego 1997, 1998). However, four of the 11 known rap genes are not followed by functional phr genes, suggesting that these are not involved in quorum-sensing processes. Nevertheless, it cannot be excluded that these four Raps respond to Phr peptides encoded in other operons.

Our present studies indicate that at least three chromosomally encoded Rap-Phr systems of B. subtilis 168 are involved in the transcriptional control of the aprE gene. In fact, both the extracellular addition of synthetic PhrA, PhrE and PhrF pentapeptides, and the disruption of the individual rapA, rapE or rapF genes caused an increase in aprE transcription. Finally, the secretion of proteolytic enzymes increased in a strain carrying a degU32 (Hy) mutation, in which the rapA gene was disrupted. These data show that chromosomally encoded Rap-Phr systems can be engineered for the improved production of extracellular proteases. Furthermore, these data imply that quorum-sensing systems that are important for bacterial adaptation under natural conditions, can decrease production yields when used for production processes.

### MATERIALS AND METHODS

#### Plasmids, bacterial strains and media

Table 1 lists the plasmids and bacterial strains used. TY medium (tryptone/yeast extract) contained Bacto tryptone.

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**Table 1** Plasmids and bacterial strains

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(1%), Bacto yeast extract (0.5%) and NaCl (1%). Extracellular proteolytic activity was tested on TY agar (1.5%) with 1% of skim milk. When required, media for \textit{B. subtilis} were supplemented with kanamycin (Km; 5 \mu g ml\(^{-1}\)) or spectinomycin (Sp; 100 \mu g ml\(^{-1}\)) or erthyromycin (1 \mu g ml\(^{-1}\)). Phr peptides were obtained from Genencor International (Palo Alto, CA, USA), and added to the medium in a 0.1 or 10-\mu M concentration when desired.

**DNA techniques**

Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis and transformation of \textit{E. coli} were carried out as described by Sambrook et al. (1989). Enzymes were from Roche Diagnostics. \textit{Bacillus subtilis} was transformed as described by Bron and Venema (1972). For a high reliability PCR, Pwo polymerase (Roche Diagnostics) was used.

To construct \textit{B. subtilis} \textit{phrE} (Fig. 1a), a fragment comprising the \textit{phrE} gene was amplified with the primers 5'-E-XbaI (5'-TAT ATC TAG ATC GAA AGG GGA ATG CAT GTA TGA AAT C-3') and 3'E-BamHI (5'-TTT AGG ATC CAG CGC TTA AAC AAG GAA TTC ATG AGT AGG TGC G-3'). The amplified fragment was cleaved with XbaI and BamHI, and ligated into the SpeI and BamHI sites of px (Kim et al. 1996), resulting in plasmid pHR-E. Next, the cassette containing the xylose-inducible \textit{phrE} gene of pHR-E was inserted into the \textit{amyE} gene of \textit{B. subtilis} 168 by replacement recombination.

To construct \textit{B. subtilis} \textit{phrF} (Fig. 1a), a fragment comprising the \textit{phrF} gene was amplified with the primers 5’-F-XbaI (5’-TTA ATC TAG AAG GAG GAG TTT AGG ATC CAG CGC TTA AAC AAG GAA TTC ATG AGT AGG TGC G-3') and 3’-F-BamHI (5’-TAA TGG ATC CTT AGG GTA TGA GGT TTT ATG AAA TTTG-3'). The amplified fragment was cleaved with XbaI and BamHI, and ligated into the SpeI and BamHI sites of pX (Kim et al. 1996), resulting in plasmid pHRF. Next, the cassette containing the xylose-inducible \textit{phrF} gene of pHRF was inserted into the \textit{amyE} gene of \textit{B. subtilis} 168 by replacement recombination.

To construct \textit{B. subtilis} \textit{rapA}-Cm (Fig. 1b), a fragment comprising the \textit{rapA} gene was amplified with the primers \textit{rap} A-1 (5’-AGA ATT CAA GCT TAG AAG ACA TCG AAG GG-3') and 3' -rapA-1 (5' -ATC GGA TCC TTA GTG TTG ATT GCG TGC CGC-3'). The amplified fragment was cleaved with HindIII and BamHI, and ligated into the HindIII and BamHI sites of pUK21, resulting in pUKA. The \textit{rapA} gene of the latter plasmid was interrupted by a chloramphenicol resistance marker, resulting in plasmid pUKA-Cm. Next, the chromosomal \textit{rapA} gene of \textit{B. subtilis} was disrupted by replacement recombination of pUKA-Cm with the \textit{rapA} gene of \textit{B. subtilis} 168.

To construct \textit{B. subtilis} \textit{rapE}-Sp (Fig. 1b), a fragment comprising the \textit{rapE} gene was amplified with the primers \textit{rapEb} (5’-CCA TCG ATG GAT CCC ACA TCA GCTGAA GTG GGA ATG-3') and \textit{rapE} (5’-CGG GAT CCA TCGATG GTT GCC TCC TAA CAA TGC CAG C-3'). The amplified fragment was cleaved with XbaI, and ligated into the corresponding sites of pUC7, resulting in pUC5E. The \textit{rapE} gene of the latter plasmid was interrupted by a spectinomycin resistance marker, resulting in plasmid pUC5E-Sp. Next, the chromosomal \textit{rapE} gene of \textit{B. subtilis} was disrupted by replacement recombination of pUC5E-Sp with the \textit{rapE} gene of \textit{B. subtilis} 168.

To construct \textit{B. subtilis} \textit{rapF}-Sp (Fig. 1b), a fragment comprising the \textit{rapF} gene was amplified with the primers \textit{rapF}-1 (5’-AGT CTC TAG ATA TGA CAT ATA TAG AAT TAT GGG AGG GAG G-G-3') and \textit{rapF}-2 (5’-ATG CCG ATC CGT CGC ACT CGA CGT TTG GCC TAA CTC CG). The amplified fragment was cleaved with XbaI and BamHI, and ligated into the XbaI and BamHI sites of pUK21, resulting in pUKF. A 96 bp internal HindIII fragment from the \textit{rapF} gene of the latter plasmid was replaced by a spectinomycin resistance marker, resulting in plasmid pUKF-Sp. Next, the chromosomal \textit{rapF} gene of \textit{B. subtilis} was disrupted by replacement recombination of pUKF-Cm with the \textit{rapF} gene of \textit{B. subtilis} 168.

In some experiments, the \textit{degU32} (Hy) mutation, or the transcriptional \textit{aprE-lacZ} fusion, or both, were introduced in \textit{B. subtilis} \textit{rapA} by transformation with chromosomal DNAs of the \textit{B. subtilis} strains 168 \textit{degU32} (Hy) and/or 168 AL (Table 1). Constructed strains were selected on plates containing the appropriate antibiotic and checked by PCR analysis for the correct integration of plasmids into the chromosome.

**\beta-\text{Galactosidase assays**}

Overnight cultures were diluted to an optical density (O.D.600) of 0.1 in fresh TY medium and supplemented with Phr pentapeptide(s) when desired (sequences of chemical synthesized PhrA, -C, -E, -F, -G, -I, and -K are bold and underlined in Table 2). Samples for O.D.600 readings and \beta-galactosidase activity determinations were taken at hourly intervals. The assays and the calculations of \beta-galactosidase activities (expressed as units per O.D.600) of the samples were performed as described by Miller (1982). Experiments were repeated at least three times and the relative effects within one experiment were reproducible; the results of only one of these experiments are presented.

**Protease activity assays**

Medium of overnight cultures grown in TY medium was diluted 10-fold in buffer containing 10 mM Tris (pH 6.8);
Fig. 1 Schematic presentation of the construction of *Bacillus subtilis* XphrE, XphrF, ΔrapA-Cm, ΔrapE-Sp, and ΔrapF-Sp. (a) Xylose-inducible phrE and phrF genes were inserted in the amyE gene by replacement recombination (see 'Materials and methods'). *amyE′*, 3′-truncated *amyE* gene; *amyE′*, 5′-truncated *amyE* gene; Cmr, chloramphenicol resistance marker; *xylR*, repressor of *xylA* promoter; *P*_{xylA}, xylose-inducible *xylA* promoter. (b) The chromosomal rapA, rapE and rapF genes were disrupted with a chloramphenicol (Cmr), and spectinomycin (Sp′) resistance markers by replacement recombination (see 'Materials and methods'). Only restriction sites relevant for the constructions are shown (b; BglII; N, *Hin* dll II; H, NsI). The relative positions of the phrA, phrE and phrF genes are indicated according to Kunst *et al.* (1997). *rapA′*, *rapE′*, *rapF′*, 3′-truncated *rapA*, *rapE*, *rapF* genes; *rapA′*, *rapE′*, *rapF′*, 5′-truncated *rapA*, *rapE*, *rapF* genes.
Table 2 Pre-Phr proteins and their active pentapeptides*. Hydrophobic domains of the putative signal peptides are indicated in grey shadings†, predicted signal peptidase recognition sequences‡ in italics. Putative processing sites are indicated with an arrow. The sequences of synthetic Phr pentapeptides used in this study are underlined and in bold

| PhrA | MKSKVMSGLLAVAVGESAFTQVMFA \(\downarrow\) GETANTEGKTHIA \(\downarrow\) ARNQT |
| PhrC | MKLKSKPVIICAAAIIIPTAAGVSNJIA \(\downarrow\) EALDFHYV \(\downarrow\) ERGMT |
| PhrE | MKSKFLFSLAVLGLGAFGSQNGEMKEDA \(\downarrow\) SRNVT \(\downarrow\) LAPTHEFLV |
| PhrF | MKLKSFLSCLALSTVFVGAATIJA \(\downarrow\) NAPTHQJEVA \(\downarrow\) QRGMI |
| PhrG | MKRFILGAGAVAVILSGGWFA \(\downarrow\) DHQTHSQEMKVA \(\downarrow\) EKMI |
| PhrI | MKISRLFLAALVILSSPSVTYFLQSDHIENKVA \(\downarrow\) DVRGA |
| PhrK | MKKLVLCYSLAVLSGLVA \(\downarrow\) LTQLSTDSPSNIQVA \(\downarrow\) ERPVG \(\downarrow\) G |

*Sequences were obtained from SubtiList (http://genolist.pasteur.fr/SubtiList/).
†Hydrophobic regions were predicted with the TMHMM algorithm (http://www.cbs.dtu.dk/services/TMHMM; see Tjalsma et al. 2000).
‡Signal peptidase recognition sequences were predicted with the SignalP algorithm (http://www.cbs.dtu.dk/services/SignalP-2.0; see Tjalsma et al. 2000).

100 mM NaCl and 1·4 mg ml⁻¹ azo dye Impregnated collagen (Sigma), and incubated for 1 h at 37°C. Next, the release of the dye was measured by optical density readings at 520 nm as a quantification of the proteolytic activity.

RESULTS

Chromosomally encoded Phr peptides are involved in aprE Transcription Regulation

To determine whether, like the plasmid-encoded Rap60-Phr60 system (Koetje et al. 2003), chromosomally encoded Rap-Phr systems are involved in aprE transcription, B. subtilis 168 AL (bearing an aprE-lacZ transcriptional fusion), was grown in TY medium supplemented with 10 µM of synthetic PhrA, -C, -E, -F, -G, -H, -I, or -K pentapeptides (see Table 2). The concentration of 10 µM Phr peptide was used as previous studies have shown that at this Phr60 concentration – added at the beginning of growth of the culture – an optimal Rap60 inhibition was observed. This led to the complete rescue of aprE-lacZ transcription in a strain expressing the plasmid-borne Rap60 (Koetje et al. 2003). As shown by β-galactosidase assays performed with cells harvested in the postexponential growth phase (Fig. 2a), PhrE and PhrF stimulated aprE transcription (±135% of wildtype); PhrG, PhrA, PhrK and PhrI weakly stimulated aprE transcription (±110–120% of wildtype); whereas PhrC had a clear negative effect on aprE transcription (±60% of wildtype). Growth rates and cell densities were not significantly affected by the presence of Phr peptides in the culture medium (data not shown). These results suggest that the transcription of aprE is regulated by the action of several Rap-Phr systems. To further study the effect of the PhrE peptide on the transcription of aprE during growth, synthetic PhrE was added at two concentrations (1 and 10 µM) to the medium of B. subtilis AL. PhrE was chosen because it caused a significant increase in aprE transcription (see Fig. 2a), and it shows the highest level of homology with the plasmid-encoded Phr60 peptide (data not shown), which was shown to be involved in aprE transcription (Koetje et al. 2003). The results show that aprE transcription was improved by the addition of PhrE only during the postexponential growth phase in a PhrE concentration-dependent manner (Fig. 2b).

To analyse whether intracellular overproduction of pre-PhrE and pre-PhrF can increase aprE transcription, B. subtilis XphrE AL (aprE-lacZ; xylose-inducible production of pre-PhrE) and B. subtilis XphrF AL (aprE-lacZ; xylose-inducible production of pre-PhrF), were constructed (see ‘Materials and methods’ section; Fig. 1a) and, together with B. subtilis AL (bearing only aprE-lacZ), grown in TY medium supplemented with 1% xylose. As shown by β-galactosidase assays (Fig. 3), production of pre-PhrE and pre-PhrF had a stimulatory effect on aprE transcription during the postexponential period of the growth phase. Growth rates and cell densities were not significantly affected by the induction of the phrE/F genes by xylose, and no significant effects were observed on aprE transcription when these cells were grown in the absence of xylose (data not shown). These results show that overproduction of pre-PhrE and pre-PhrF can improve aprE transcription, most likely by inhibiting RapE and RapF, respectively.

Taken together, these results indicate that the Phr quorum-sensing peptides are modulators of aprE transcription. Importantly, these data suggest that, especially, RapE and RapF are involved in the repression of aprE transcription.

RapA, RapE and RapF exert negative control on aprE transcription

To further investigate whether transcription of aprE is controlled by the chromosomally encoded Rap-Phr systems of B. subtilis, rapA, rapE and rapF disruption mutants were constructed (see ‘Materials and methods’ section). These genes were selected because of the strongest stimulatory effect of PhrE and PhrF on aprE transcription (see Fig. 2a),
and because rapA, like rapE, is known to be involved in the cell density–controlled regulation of the phosphorelay (Perego 1998; Tzeng et al. 1998). To study the effects of rapA, rapE and rapF mutations on aprE transcription, these mutations were introduced into B. subtilis AL carrying the aprE-lacZ fusion. Next, B. subtilis AL, B. subtilis ΔrapA AL, B. subtilis ΔrapE AL, and B. subtilis ΔrapF AL were grown at 37°C in TY medium. As shown in Fig. 4 aprE transcription was improved during the postexponential growth phases by each of these mutations. Surprisingly, considering the modest effect of PhrA (see Fig. 2a), disruption of rapA had the strongest effect on aprE transcription of all three tested rap mutants. Apparently,

Fig. 2 Effects of Phr pentapeptides on aprE transcription. (a) The transcription of the aprE-lacZ gene fusion in Bacillus subtilis AL grown in TY medium at 37°C in the presence of 10 µM of one of the following pentapeptides (see Table 2): PhrA (A), PhrC, (C), PhrE (E), PhrF (F), PhrG (G), PhrI (I) or PhrK (K) or in the absence of pentapeptides (–), was measured 4 h after the transition point between the exponential and postexponential growth phases. β-Galactosidase activities were determined in units per O.D.600 (% of parental; aprE-lacZ transcription level of the strain grown in the absence of Phr peptides (parental) is set to 100%). Values are the average of three individual experiments, and the standard deviation is indicated by error bars. (b) Time courses of the transcription of the aprE-lacZ gene fusion in B. subtilis AL grown in TY medium at 37°C, without PhrE peptide (○), and with 1 µM PhrE (■), or 10 µM PhrE (▲) peptide added to the medium were determined. β-Galactosidase activities were determined in units per O.D.600. Zero time (T0) indicates the transition point between the exponential and postexponential growth phases.
the addition of equal Phr peptide concentrations does not result in similar inhibition rates of the corresponding Raps. Although this observation indicates that our Phr-inhibition approach to select rap target mutations might not yield the best rap target genes, we continued with our best candidate: rapA. This mutation caused an increase of about 120–200% of wildtype, whereas disruption of rapE and rapF both resulted in about 110–150% increase of aprE transcription. Growth rates and cell densities were not significantly affected by rap disruptions (data not shown). This indicates that RapA, RapE and RapF are repressors of aprE transcription, by the dephosphorylation of SpoOF~P (Perego 1998; Tzeng et al. 1998) an/or via other unknown mechanisms. Moreover, this implies that disruption of these Rap-Phr quorum-sensing systems may improve protease production by B. subtilis.

Improved extracellular protease production by a ΔrapA degU32 (Hy) strain

To test whether engineering of Rap-Phr systems can be used for improved protease production, we introduced a rapA mutation in a B. subtilis strain carrying a degU32 (Hy) mutation. The latter mutation results in stable DegU~P, causing hyper-transcription of many protease genes, such as aprE (Olmos et al. 1997). In wild-type strains, DegU is phosphorylated via DegS, which senses certain specific signals during postexponential growth phases (Msaedek et al. 1990; Kunst et al. 1994). The degU32 (Hy) mutation appears to be present in most Bacillus production strains. It may be a common feature for such strains to achieve high production yields during industrial fermentation (E. Ferrari and S. Causey, unpublished observations). To study whether disruption of rapA improves protease secretion in a strain with a degU32 (Hy) mutation, first the aprE-lacZ fusion was introduced in this chromosomal background. Next, B. subtilis degU32 (Hy) AL and B. subtilis ΔrapA degU32 (Hy) AL were grown at 37°C in TY medium. As shown by β-galactosidase assays (Fig. 5a), disruption of rapA had a positive effect on aprE transcription during postexponential growth. To evaluate extracellular protease production of these strains, a plate assay was used. The halo around colonies of B. subtilis ΔrapA degU32 (Hy) was larger than that around colonies of cells containing only the degU32 (Hy) mutation (Fig. 5b). Similarly, the proteolytic activity in the liquid medium of overnight cultures of B. subtilis ΔrapA degU32 (Hy) was about 135% of that in B. subtilis degU32 (Hy) (Fig. 5b). In the protease production assays (Fig. 5b), the activity of at least six other extracellular proteases is measured (see Koetje et al. 2003), which makes a direct correlation with aprE-lacZ transcription assays (Fig. 5a) not possible. Taken together, these results show that extracellular protease production can be improved by the engineering of Rap-Phr quorum-sensing systems, as exemplified with the disruption of the rapA gene.
DISCUSSION

Our present and previous (Koetje et al. 2003) studies show that Rap-Phr systems are involved in the cell density-controlled regulation of extracellular protease production in B. subtilis. More specifically, they are involved in the repression of protease-encoding genes, such as aprE. This property of the Rap-Phr systems may be important for the fitness of B. subtilis under natural conditions, as it may prevent protease production under nutrient-rich conditions, thereby making more efficient use of the available energy sources. However, the present results indicate that these Rap-Phr systems are not the major factors that repress gene expression at low cell densities, as aprE transcription does not start earlier during growth when rap genes are disrupted or Phr peptides are overproduced or added to the medium. In fact, an increase in aprE transcription was only seen during the postexponential growth phase, suggesting that the negative regulators AbrB, SinR and ScoC are still, to some extent, bound to the aprE promoter. A possible model for Rap-Phr-mediated aprE transcription is depicted in Fig. 6. RapA and RapE are able to dephosphorylate SpoOF~P, a component of the phosphorelay (SpoOF-SpoOB-SpoOA; Jiang et al. 2000; Koetje et al. 2003), thereby lowering the cellular levels of SpoOA~P. At present, we do not know the exact role of RapF and PhrF in aprE transcription (Fig. 6, indicated by question mark). Our present results may suggest that RapF is also involved in the dephosphorylation of one of the components of the phosphorelay. Nevertheless, we cannot exclude that effects of the rapF mutations on aprE transcription are indirectly linked to the phosphorelay, or even completely independent of this relay. For example, RapF might be able to bind to, and inhibit the activity of, the response regulator DegU, as was recently shown for RapG (Ogura et al. 2003). Similar to the disruption of rapF (this study), disruption of rapG (Ogura et al. 2003) had an enhancing effect on aprE transcription. As indicated in Fig. 6, extracellular accumulated PhrA and PhrE peptides can be taken up from the environment by the Opp system. This results in the inhibition of RapA and RapE, and thus elevated SpoOA~P levels. Finally, this results in repression of the genes coding for AbrB, SinR and ScoC, all of which repress aprE transcription by binding to the aprE promoter region (Jan et al. 2000; Caldwell et al. 2001). When this repression is (partially) removed, aprE transcription can be stimulated by positive regulators, such as DegU~P. The fact that the

Fig. 6 Model for Rap-Phr-mediated regulation of aprE transcription. RapA and RapE are able to dephosphorylate SpoOF~P, a component of the phosphorelay (SpoOF-SpoOB-SpoOA), thereby lowering the cellular levels of SpoOA~P. In contrast, the target of RapF is still unknown. At high cell densities, sufficient amounts of PhrA, -E, and -F have been accumulated in the medium to be taken up in sufficient amounts by the Opp system. This results in RapA, -E, -F inhibition, more phosphorylated SpoOA and, eventually, increased aprE transcription as the cellular levels of AbrB, ScoC and SinR go down (Koetje et al. 2003). After entry into the postexponential growth phase aprE transcription is stimulated by DegU~P. Notably, extracellular PhrC inhibits aprE transcription, possibly because of higher cellular levels of ComA~P, causing an increased transcription level of the rapA, rapE and rapF genes.
PhrC pentapeptide represses aprE transcription can be explained by the fact that this peptide inhibits RapC that acts on ComA–P (Solomon et al. 1996). Thus, addition of PhrC results in more phosphorylated ComA, resulting in elevated expression level of the rapA, rapE (Jiang et al. 2000), and rapF (Ogura et al. 2001) genes, eventually leading to more dephosphorylation of Spo0F by RapA, RapE and possibly RapF (Fig. 6). The latter regulation pathway seems to be quite important, as disruption of the oligopeptide uptake system (Opp) results in an 1.5–2-fold increased expression of aprE during the postexponential growth phase (unpublished observations). As PhrC seems the only Phr peptide taken up by the Opp system with an inhibitory effect on aprE transcription, this effect is likely to be attributed to the lack of PhrC internalization. However, it should be noted that the concentration of a certain Phr peptides in the growth medium is an important determinant for the eventual effect on gene expression. For example, low concentrations of PhrC stimulated competence, whereas high concentrations of PhrC stimulated the ability of cells to sporulate at low cell densities (Solomon et al. 1996). Thus, it has to be kept in mind that the Phr concentrations used in this study might only reveal part of their activity spectrum on aprE transcription.

In conclusion, quorum-sensing by Rap-Phr systems is an intertwined and highly regulated process important for the fine tuning of transition-associated phenomena, including aprE expression. However, as exemplified by the fact that mutations in the Rap-Phr systems can increase aprE transcription, fine-tuning is not always beneficial for the yield of large-scale fermentations. In this respect it is worth mentioning that aprE transcription was not further increased in multiple rap mutants nor by addition of mixtures of Phr peptides (unpublished observations). This supports the notion that Rap-Phr systems are fine-tuning systems, rather than key regulators, of postexponential gene transcription. Thus, engineering of Rap-Phr systems of B. subtilis can be exploited for the production of industrial proteins such as AprE. Although the levels of aprE transcriptional increase are moderate in the model strain B. subtilis 168, this approach may be feasible to increase the yield of Bacillus production strains, and possibly other production organisms endowed with quorum-sensing systems (see DeLisa et al. 2001).

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