Regulated Secretion of a Protease Activates Intercellular Signaling during Fruiting Body Formation in *M. xanthus* 

Anne Rolbetzki,1 Meike Ammon,1 Vladimir Jakovljevic,1 Anna Konovalova,1 and Lotte Søgaard-Andersen1,*

1Department of Ecophysiology, Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch Strasse, 35043 Marburg, Germany
*Correspondence: sogaard@mpi-marburg.mpg.de
DOI 10.1016/j.devcel.2008.08.002

**SUMMARY**

In response to starvation *Myxococcus xanthus* initiates a developmental program that culminates in fruiting body formation. There are two morphogenetic events in this program, aggregation and sporulation, which are temporally and spatially coordinated by the contact-dependent intercellular C-signal protein (p17). p17 is generated by proteolytic cleavage of the p25 precursor protein, which accumulates in the outer membrane of vegetative and starving cells. However, p17 generation is restricted to starving cells. Here we identify the subtilisin-like protease PopC that is directly responsible for cleavage of p25. PopC accumulates in the cytoplasm of vegetative cells but is selectively secreted during starvation coinciding with the generation of p17. Consequently, p25 and PopC only encounter each other in starving cells. Thus, restriction of p25 cleavage to starving cells occurs by a compartmentalization mechanism that depends on selective secretion of PopC during starvation. Our results provide evidence for regulated proteolysis via regulated secretion.

**INTRODUCTION**

Intercellular signaling systems allow bacteria to control cellular activities in response to cell density or cell position (Bassler and Losick, 2006). In systems communicating cell density, the signals are small diffusible molecules and signal transmission does not involve direct cell-cell contact, whereas in systems communicating cell position, the signals are cell-surface-associated proteins and signal transmission depends on direct cell-cell contact (Bassler and Losick, 2006). An example of contact-dependent signal transmission is provided by the intercellular C-signal during fruiting body formation in *Myxococcus xanthus*. Fruiting body formation is initiated in response to starvation and includes two morphogenetic events, aggregation of cells into fruiting bodies and sporulation. These two events are coordinated temporally and spatially. Aggregation precedes sporulation and only cells that have accumulated inside fruiting bodies undergo sporulation. The intercellular C-signal has a key function in this coordination (Kim and Kaiser, 1991; Kruse et al., 2001; Li et al., 1992). The C-signal is a 17 kDa protein (p17) (Kim and Kaiser, 1990a; Lobedanz and Søgaard-Andersen, 2003), which is anchored in the outer membrane (Lobedanz and Søgaard-Andersen, 2003) and exposed on the cell surface (Shimkets and Rafiee, 1990). Consistent with the cell-surface localization of p17, C-signal transmission is contact-dependent (Kim and Kaiser, 1991; Kruse et al., 2001; Li et al., 1992). According to current models, the regulated accumulation of p17 during starvation (Kruse et al., 2001) together with the contact-dependent signaling mechanism helps to guarantee the correct temporal and spatial coordination of aggregation and sporulation (Julien et al., 2000; Kruse et al., 2001).

p17 is generated by proteolytic cleavage of a 25 kDa precursor protein (p25) encoded by the *csgA* gene resulting in the removal of 8 kDa from the N terminus of p25 (Lobedanz and Søgaard-Andersen, 2003). p25 is anchored in the outer membrane (Lobedanz and Søgaard-Andersen, 2003), exposed on the cell surface (Shimkets and Rafiee, 1990), and present in vegetative as well as starved cells (Kruse et al., 2001). p17, however, only accumulates in starved cells (Kruse et al., 2001). To elucidate the mechanism by which *M. xanthus* restricts p17 generation to starving cells, we focused on identifying the protease that cleaves p25. Here, we identify the PopC protease as directly responsible for cleavage of p25. PopC accumulates in the cytoplasm of vegetative and starving cells; however, PopC is selectively secreted in response to starvation. Accordingly, p25 and PopC are only present in the same cell compartment in starving cells. These observations suggest that restriction of p25 cleavage to starving cells is controlled by a compartmentalization mechanism that depends on the selective secretion of PopC during starvation.

**RESULTS**

**Identification of PopC**

Addition of *M. xanthus* cell extract to a purified MalE-p25 protein results in cleavage of p25 to generate p17, and this cleavage is inhibited by the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (Lobedanz and Søgaard-Andersen, 2003). These observations, taken together with the observations that both p25 and p17 are anchored in the outer membrane and...
exposed on the cell surface, led us to hypothesize that p25 is cleaved by a secreted serine protease. Analysis of the *M. xanthus* genome resulted in the identification of 32 genes likely to encode secreted serine proteases or serine proteases associated with the cell envelope (Table S1, see the Supplemental Data available online). Based on the predicted subcellular localization and PMSF inhibition profile of these 32 proteases (Table S1), we narrowed our focus to 20 genes encoding 10 trypsin-like and 10 subtilisin-like proteases. Because the p25-cleaving activity is upregulated in starving cells (Lobedanz and Søgaard-Andersen, 2003), we hypothesized that transcription of the relevant protease gene is upregulated during starvation. Genome-wide expression profiling experiments (Shi et al., 2008) showed that five (MXAN0206, MXAN1501, MXAN4277, MXAN7209, and MXAN7328) of the 20 candidate genes fulfill this criterion (Table S1).

To determine whether any of these five genes are involved in fruiting body formation, we generated insertion mutations in each of the five genes in the wild-type strain DK1622. Similar to a csgA mutant, which is unable to synthesize p25, MXAN0206 mutant cells (SA2314) did not show any signs of fruiting body formation even after 120 hr of starvation in submerged culture (Figure 1A). In contrast, wild-type cells and the remaining four mutants formed fruiting bodies after 24 hr (Figure 1A and data not shown). Moreover, sporulation in the MXAN0206 mutant was strongly reduced (<0.0005% of wild-type levels), as is sporulation in a csgA mutant. Therefore, we focused on the MXAN0206 gene, from hereon designated *popC*, for protease required for processing of C-signal precursor.

*PopC* consists of a unique N-terminal extension (residues 1-175) and a C-terminal domain (residues 176-475), which is similar to subtilisin-like proteases (Figure 1B). The C-terminal domain contains the Asp-His-Ser catalytic triad characteristic of subtilisin-like proteases and the conserved Asn residue that forms the oxyanion hole and is important for stabilization of the transition state (Figure 1B; Siezen and Leunissen, 1997). In the catalytic triad, Ser is the active site residue responsible for peptide bond cleavage. Although *PopC* lacks a signal peptide (Table S1), we initially included *PopC* among serine proteases likely to be secreted because most bacterial subtilisins are secreted (Siezen and Leunissen, 1997).

*M. xanthus* cells move by gliding motility using two motility systems referred to as the A- and S-motility systems (Søgaard-Andersen, 2004) and motility defects can cause defects in fruiting body formation (Hodgkin and Kaiser, 1979). When assayed in parallel with wild-type cells and mutants with defects in A-motility (A- S ) or S-motility (A- S ), the *popC* mutant formed colonies similar to that of wild-type with flares and single cells at the colony edge (Figure 1C). Thus, the *popC* mutation does not affect development indirectly through an effect on motility.

*PopC* is cotranscribed with the downstream gene MXAN0207 (data not shown; Figure 1D). The *popC* mutation was generated by integration of plasmid pAB3 in *popC* (Figure 1D). To rule out that the developmental defects in the *popC* mutant were caused by a polar effect on MXAN0207, we carried out genetic complementation experiments. Plasmid pAB25, which contains the *popC* allele including the native promoter (Figure 1D), was integrated at the chromosomal Mx8 phage attachment site in the *popC* mutant to generate strain SA2327. As shown in Figure 1A, the *popC* allele corrected all developmental defects caused by inactivation of *popC*, and therefore, these defects were not caused by a polar effect on MXAN0207. Moreover, in SA2327 *PopC* accumulated at the same levels as in wild-type cells (Figure 3A, see below). To determine whether *PopC* protease activity is important for fruiting body formation, we substituted the active site Ser423 residue in *PopC* with Ala (Figure 1B), and introduced the resulting plasmid pAB26 (Figure 1D) into the *popC* mutant at the Mx8 attachment site to generate strain SA2329. The *popC* (S423A) allele did not complement the developmental defects caused by the *popC* mutation (Figure 1A). The inability of the *popC* (S423A) allele to complement the *popC* mutation was not caused by differences in *PopC* protein accumulation as *PopC* (S423A) and wild-type *PopC* accumulated to similar levels, as shown by immunoblot analyses using anti-*PopC* antibodies (Figure 3A; see below). These results indicate that *PopC* is a protease that is directly required for fruiting body formation.

### PopC Directly Cleaves p25 to Generate p17

Next, we analyzed whether *PopC* is involved in p17 generation. As previously reported, p25 accumulated in vegetative wild-type cells as well as in cells starved in submerged culture, and p17 only began to accumulate after 3 hr of starvation (Figure 2A). Cells of SA2327 (*popC*/*popC*) displayed an accumulation profile of p25 and p17 similar to that in DK1622 wild-type. In the *popC* and *popC* (S423A) mutants, p25 accumulated as in the wild-type, but p17 was not detected. In all four strains, p25 was only detected in the outer membrane (data not shown). Thus, *PopC* is important for cleavage of p25 after export of p25 to the outer membrane.

Addition of total cell extract from starving wild-type cells to a MalE-p25 protein results in formation of p17 (Lobedanz and Søgaard-Andersen, 2003). To determine whether *PopC* is required for cleavage of p25 under these conditions, we added total cell extract prepared from wild-type, *popC*, and *popC* (S423A) cells starved on TPM agar for 9 hr to MalE-p25. As previously reported, wild-type cell extract cleaved MalE-p25 to generate a 17 kDa protein that was recognized by antibodies against the C-terminal part of p25 but not by antibodies against the N-terminal part of p25, thus confirming the identity of p17 (Figure 2B). In contrast, addition of cell extracts of the *popC* and *popC* (S423A) mutants to MalE-p25 did not result in the generation of p17 (Figure 2B). Thus, *PopC* is required for p25 cleavage also under these conditions.

To determine whether *PopC* directly cleaves p25, we purified *PopC*-His6 and *PopC* (S423A)-His6 fusion proteins by affinity chromatography. Addition of *PopC*-His6 to MalE-p25 resulted in generation of p17, whereas addition of *PopC* (S423A)-His6 to MalE-p25 did not (Figure 2C). The assay in Figure 2C required long incubation times (24 hr) and a relatively high concentration of *PopC*-His6 (4.7 μM) compared to MalE-p25 (2.5 μM). *PopC*-His6 was purified from inclusion bodies and we speculate that the fraction of active enzyme may be low, thus, causing the low activity. The specificity of *PopC* cleavage of p25 in vitro was verified by showing that the subtilisin-like protease Proteinase K (final concentration 3.5 nM) completely degraded MalE-p25 in the in vitro protease assay (data not shown). Thus, *PopC* directly cleaves p25 to generate p17.
PopC Is Specifically Secreted during Starvation

We analyzed PopC accumulation, localization, and secretion by immunoblotting using antibodies raised against full-length PopC-His6. In wild-type, anti-PopC antibodies recognized a protein similar in size to that of the calculated molecular mass of PopC (50.8 kDa), which was not present in the \textit{popC} mutant (Figure 3A) arguing that the antibodies are specific. In control experiments, full-length PopC-His6 and a His6-tagged PopC protease domain (residues 176–475 of PopC) migrated according to their calculated molecular masses (data not shown), arguing that the PopC protein detected in wild-type \textit{M. xanthus} cells corresponds to full-length PopC. PopC accumulated in vegetative cells, and PopC accumulation decreased during starvation in submerged culture (Figure 3A).

To determine to which cell compartment PopC is localized, two different cell fractionation procedures were used. To determine whether PopC is associated with the inner or outer membrane, extracts of wild-type \textit{M. xanthus} cells were fractionated in the presence of protease inhibitors into fractions enriched for inner membrane proteins, outer membrane proteins, and...
soluble proteins, which include cytoplasmic and periplasmic proteins. In both vegetative cells and cells starved for 3 hr, PopC was only detected in the fraction enriched for soluble proteins, and not detected in the fractions enriched for inner and outer membrane proteins (Figure 3B). Control experiments with proteins localized in the cytoplasm (PilC), inner membrane (PilT), or outer membrane (PilQ) verified that the fractionation procedure functioned correctly (Figure 3B). To discriminate whether PopC is localized to the cytoplasm or periplasm, cells were fractionated in the presence of protease inhibitors using an osmotic shock fractionation procedure, which allows the separation of periplasmic from cytoplasmic proteins. In both vegetative cells and cells starved for 3 hr, PopC was only detected in the cytoplasmic fraction (Figure 3C). Control experiments with antibodies against PilT, PilC, and PilQ verified that the fractionation procedure functioned correctly (Figure 3C). From these experiments we conclude that PopC is a cytoplasmic protein.

To determine whether PopC is secreted, we isolated the cell-free supernatant from vegetative cells and from cells starved for 3 hr in suspension (Kuspa et al., 1986). As shown in Figure 3D, PopC was detected in the supernatant from starving cells but not in that from vegetative cells. These data suggest that PopC is a secreted protein and that PopC is specifically secreted in response to starvation. To test this hypothesis in more detail, we analyzed PopC accumulation in the supernatant of wild-type cells at different time points during starvation in suspension. When cells were starved in the absence of protease inhibitors, PopC accumulation decreased in total cell extracts, whereas PopC only accumulated at low levels in the supernatant (Figure 3E). However, when cells were starved in the presence of protease inhibitors, PopC accumulation in the supernatant increased gradually during development and this increase was paralleled by decreased PopC accumulation in total cell extracts (Figure 3E). To verify that accumulation of PopC in the supernatant from starving cells was due to secretion and not cell lysis, the accumulation of PilT, PilC, and PilQ in the supernatant was analyzed in cells that had been starved for 6 hr in suspension in the presence of protease inhibitors. PilT, PilC, and PilQ were only detected in total cell extract and not in the supernatant (Figure 3E). These observations confirm that PopC accumulation in the supernatant is specifically due to secretion. As expected, p17 only accumulated when cells were starved in the absence of protease inhibitors (Figure 3E). Taken together these analyses show that PopC is synthesized in vegetative cells, and in these cells PopC localization is restricted to the cytoplasm. In starving cells, however, PopC secretion is activated and PopC gradually secreted and rapidly degraded in the absence of protease inhibitors.

The rapid degradation of secreted PopC led us to speculate that PopC preferentially acts in cis, i.e., PopC secreted by a cell only cleaves p25 on that cell. To address this hypothesis, csgA and popC mutant cells were mixed and starved at a 1:1 ratio in submerged culture on the basis of the following logic: if PopC acts in trans, then PopC secreted by csgA mutant cells should cleave p25 made by popC mutant cells. However, p17 was not detected in immunoblots of the mixed cell extracts (Figure 2A). In control experiments, secretion of PopC by csgA cells was verified (data not shown). These observations strongly suggest that PopC preferentially acts in cis.

Figure 2. PopC Directly Cleaves p25

(A–C) Isolated protein was separated by SDS-PAGE and analyzed by immunoblotting. Positions of p25 and p17 are indicated. (A) PopC is required for p17 synthesis in vivo. Cells were starved for the indicated periods in submerged culture. Total cell extract from 10^8 cells was added per lane and analyzed using antibodies against full-length p25. (B) PopC is required for p25 cleavage in vitro. Total cell extracts (ext.) were isolated after 9 hr of starvation on TPM agar from the indicated strains and 3.75 μg of total protein incubated separately either alone or with MalE-p25 (final concentration 2.5 μM). The first lane shows p25 and p17 from wild-type cells (DK1622). Proteins were analyzed using antibodies against an N-terminal peptide of p25 (Anti-PepN) or a C-terminal peptide of p25 (Anti-PepC). (C) PopC directly cleaves p25. Purified PopC-His6 and PopC^S423A-His6 (final concentration 4.7 μM) were incubated separately either alone or with MalE-p25 (final concentration 2.5 μM). The first lane shows p25 and p17 from wild-type cells (DK1622). Proteins were analyzed using antibodies as in (B).
Sporulation of csgA mutant cells is rescued by codevelopment with wild-type cells (Hagen et al., 1978). To examine whether PopC is only important for p17 synthesis, we tested whether sporulation of popC and csgA mutant cells could be rescued by codevelopment with wild-type cells. In these experiments, wild-type cells and mutant cells were mixed and starved at a 1:1 ratio in submerged culture. Sporulation of popC mutant cells was not rescued by codevelopment with wild-type cells, whereas sporulation of csgA mutant cells was rescued as expected (data not shown). These observations suggest that PopC in addition to p25 cleavage is important for processing of other protein(s) that are important for development and the lack of which cause autonomous development defects.

**DISCUSSION**

The data presented here demonstrate that the subtilisin-like protease PopC is directly responsible for cleavage of p25 to generate p17. PopC is present in the cytoplasm of vegetative and starving cells and is selectively secreted during starvation, coinciding with the generation of p17. These observations lead us to propose that in the case of p17 generation, p25 cleavage is restricted to starving cells by confining PopC and p25 to different cell compartments (cytoplasm and cell surface, respectively) in vegetative cells and by restricting PopC secretion to starving cells. PopC is slowly secreted during starvation and rapidly degraded upon secretion. Moreover, PopC preferentially acts in cis suggesting that PopC cleaves p25 during the secretion process or that the half-life of secreted PopC is too short to allow it to act in trans. The slow secretion of PopC during starvation and the rapid degradation of secreted PopC would both contribute to the slow conversion of p25 to p17 and, thus, ensure the gradual accumulation of p17 important for the correct temporal and spatial coordination of aggregation and sporulation.

Regulated proteolysis is a general mechanism for the activation or inactivation of regulators in biological systems (Gottesman, 2003; Jenal and Hengge-Aronis, 2003). In bacteria, regulated proteolysis may involve regulated protease synthesis.
(Chen et al., 2006), colocalization of protease and substrate in the cytoplasm (McGrath et al., 2006), or regulation of protease activity (Ades et al., 1999). In the case of the regulated proteolytic cleavage of p25, our data suggest that the restriction of p25 cleavage to starving cells is controlled by a compartmentalization mechanism that depends on the selective secretion of PopC during starvation. This mechanism is reminiscent of the regulated secretion of the subtilisin-like protease PISUB1 from exosomes into the parasitophorous vacuole leading to the regulated proteolysis of proteins required for release of the malaria parasite Plasmodium falciparum from host erythrocytes (Yeoh et al., 2007). To our knowledge, the PopC/p25 system represents the first bacterial example in which regulated proteolysis is based on regulated secretion. Likewise, to our knowledge PopC is the first reported example of a secreted protease that preferentially acts in cis.

The absence of a signal peptide in PopC and the cytoplasmic localization of PopC before secretion suggests that PopC is secreted by either a type I, III, IV, or VI secretion system (Gerlach and Hensel, 2007; Mougous et al., 2006). However, the specific secretion system involved remains to be identified. Likewise, the mechanism underlying the rapid degradation of secreted PopC remains to be elucidated. Many subtilisin-like proteases undergo autoproteolysis (Siezen and Leunissen, 1997). Interestingly, the PopC S423A active site mutant also undergoes rapid degradation upon secretion suggesting that PopC degradation does not depend on PopC protease activity (A. K. and L. S-A, unpublished data).

In eukaryotes, many prohormones are matured through proteolysis by specialized proprotein convertases (PPC), such as Kex2 in Saccharomyces cerevisiae and mammalian furins, which are subtilisin-like proteases acting in the secretory pathway (Siezen and Leunissen, 1997). Typically, different PPCs sort to different subcellular compartments (Seidah et al., 2008) and individual PPCs may sort to different subcellular compartments (Thomas, 2002). However, trafficking of individual PPCs to different subcellular compartments has not been reported to regulate prohormone processing (Seidah et al., 2008; Thomas, 2002). The finding that PopC cleaves p25 to generate p17 shows that dedicated subtilisin-like proteases that generate active intercellular signaling molecules are also present in bacteria. This finding is in agreement with the recent observation in Bacillus subtilis that generation of the intercellular signaling molecule CSF involves proteolytic cleavage of pro-CSF by secreted subtilisin and the subtilisin-like proteases Epr and Vpr (Lani- gan-Gerdes et al., 2007). Based on primary structure and domain organization, PopC, subtilisin, Epr, and Vpr are not members of the PPC subfamily of subtilisin-like proteases (Rawlings and Barrett, 1994; Rockwell and Thorner, 2004). Thus, although p25 and pro-CSF processing is functionally analogous to prohormone processing, the proteases involved belong to distinct subtilisin subfamilies.

**EXPERIMENTAL PROCEDURES**

**Growth, Development, and Motility Tests**

*M. xanthus* was grown in CTT medium and starved in submerged culture or on TPM (10 mM Tris-HCl [pH 7.6], 1 mM KPO4 [pH 7.6], 8 mM MgCl2) agar plates (Søgaard-Andersen et al., 1996). Development was followed visually using a Leica (Wetzlar, Germany) IMB/E inverted microscope and documented with a Leica DFC 350FX camera. In codevelopment experiments, cells of the two strains were mixed at a 1:1 ratio and starved in submerged culture. Spore numbers were determined as the number of germinating spores after 120 hr of starvation (Søgaard-Andersen et al., 1996). For motility assays, cells were grown in CTT, harvested, and resuspended in 1% CTT to a calculated density of 7.0 × 10^8 cells/ml. Aliquots (5 μl) were placed on 0.5% CTT, 1.5% agar, and incubated at 32°C. After 24 hr, colonies were observed in a Leica M2B stereo-microscope or in a Leica IMB/E inverted microscope and documented as described. Detailed information on how plasmids and M. xanthus strains (Table S2) were generated can be obtained from the authors. All strains constructed were confirmed by PCR.

**Immunoblot Assays**

To generate anti-PopC antibodies, PopC-His6 was purified from inclusion bodies and used to immunize rabbits using standard procedures (Sambrook et al., 1989). Antibodies against full-length p25, the N-terminal of p25, and the C-terminal of p25 have been described previously (Kruse et al., 2001; Lobedanz and Søgaard-Andersen, 2003). To analyze protein accumulation, cells were starved in submerged culture and harvested at the indicated time points. Immunoblotting followed standard procedures (Sambrook et al., 1989).

**Cell Fractionation**

Cells were either grown in liquid CTT or starved for 3 hr on TPM agar plates. Cells were fractionated using two different procedures. All fractions were analyzed by immunoblotting. As controls for proper fractionation, fractions were tested with antibodies against PilT in the cytoplasm (Jakovljevic et al., 2008), PilC in the inner membrane (V. J. and L. S-A, unpublished data), and PilQ in the outer membrane (L. Bulyha and L. S-A, unpublished data). To separate inner membrane and outer membrane proteins from soluble proteins (Lobedanz and Søgaard-Andersen, 2003), cells were resuspended in 50 mM Tris-HCl (pH 7.6) supplemented with Complete Mini Protease Inhibitor Cocktail (Roche, Mannheim, Germany) (protease inhibitors) as recommended by the supplier (equivalent to 1× concentration of protease inhibitors) and lysed by sonication. Cell debris was removed by centrifugation. The supernatants were centrifuged at 45,000 g for 1 hr at 4°C. The resulting supernatants are enriched in soluble proteins. Pellets containing the crude envelope fractions were resuspended in 50 mM Tris-HCl (pH 7.6), 2% Triton X-100 supplemented with protease inhibitors, and subjected to ultracentrifugation as described. The resulting supernatant is enriched in inner membrane proteins, and the pellet is enriched in outer membrane proteins. To separate cytoplasmic from periplasmic proteins (White et al., 2001), cells were resuspended in 20% sucrose (w/v), 50 mM Tris-HCl (pH 7.6) supplemented with protease inhibitors. After incubation for 10 min at 25°C, cells were harvested and osmotically shocked by resuspension in 4°C (50 mM Tris-HCl [pH 7.6]) supplemented with protease inhibitors. After a short incubation at 4°C, spheroplast formation was verified microscopically. Supernatants (containing periplasmic proteins) were separated from spheroplasts by centrifugation at 4,000 g for 10 min at 4°C. Spheroplasts were lysed by sonication and cleared from nonlysed cells by centrifugation at 16,000 g for 10 min at 4°C. The cleared lysate was centrifuged at 100,000 g × 1 hr at 4°C to separate soluble, cytoplasmic proteins from the insoluble, membrane-enriched fraction.

**Isolation of Secreted Proteins**

To isolate secreted proteins in the absence of protease inhibitors, cells were grown in liquid CTT to a density of 5 × 10^8 cells/ml. Cells were harvested by centrifugation and the cell-free supernatants kept at 4°C. To isolate secreted proteins from starving cells, cells were starved in suspension (Kuspa et al., 1998). Cell suspensions were incubated at 32°C with shaking for the indicated periods of time. The cell-free supernatants were filtered through a 0.22 μm sterile filter (Millipore, Schwalbach, Germany) and concentrated by ultrafiltration using a Microcon Ultracel YM-10 filter (Millipore) and analyzed by immunoblotting. To verify that the supernatants were cell-free and to confirm the absence of cell lysis, supernatants were tested with antibodies against PilT, PilC, and PilQ. To identify secreted proteins accumulating in the presence of protease inhibitors, cells were grown and starved as described, with the following modifications. After reaching a density of 5 × 10^8 cells/ml in liquid CTT medium, cells were grown for an additional 1.5 hr in liquid CTT.
supplemented with protease inhibitors before the cell-free supernatant was isolated; to isolate secreted proteins from starving cells in the presence of protease inhibitors, cell were starved as described except that protease inhibitors were added. To quantify band intensities in immunoblots, the immunoblots were scanned on a Typhoon Phosphorimager (GE Healthcare, Freiburg, Germany) and quantified using the ImageQuant software (GE Healthcare).

**Protease Assay**

Protease activity was assayed in vitro as described (Lobedanz and Søgaard-Andersen, 2003). Briefly, cells were starved on TPM agar for 9 hr, harvested, resuspended in A50 buffer (10 mM MOPS [pH 7.2], 1 mM CaCl₂, 4 mM MgCl₂, 50 mM NaCl) and lysed by sonication. Protein (5.75 µg) from cell extracts was used directly in a reaction mixture (total volume 25 µl) containing MalE-p25 (Lee et al., 1995) at a final concentration of 2.5 µM. Reactions were incubated for 2 hr at 4°C and analyzed by immunoblotting. PopC-His₆ and PopCSP₃₄₂₄₄-His₆ were expressed from pAB27 and pAB36, respectively, in E. coli Rosetta2(DE3) (Novagen, Darmstadt, Germany) in LB medium at 18°C and purified from inclusion bodies on Ni-NTA agarose as recommended by the manufacturer (QiAGEN). Purified proteins were refolded by rapid dilution in RF buffer (0.1 M Tris-HCl [pH 8.5], 1 mM DTT, 0.5 M L-arginine-HCl, 5 mM CaCl₂, 5 mM MgCl₂, 50 mM NaCl). RF buffer was exchanged with A50MC buffer (10 mM MOPS [pH 7.2], 5 mM CaCl₂, 5 mM MgCl₂, 50 mM NaCl) by ultrafiltration using a Microcon UltraCel YM-10 filter (Millipore). Purified refolded protein was assayed at a final concentration of 4.7 nM in A50MC buffer in a reaction mixture containing MalE-p25 at a final concentration of 2.5 µM. Reactions were incubated for 24 hr at 32°C and analyzed by immunoblotting. Proteinase K (Sigma-Aldrich, Munich, Germany) was assayed as described for PopC-His₆ except that the final concentration was 4.7 nM. Protein concentrations were determined using the Bio-Rad protein assay according to manufacturer's suggestions, using BSA as a standard.

**SUPPLEMENTAL DATA**

Supplemental Data include two tables and can found with this article online at http://www.developmentalcell.com/supplemental/S1534-5807(08)00326-2.

**ACKNOWLEDGMENTS**

We thank Wolfgang Garten for helpful discussions and Jürgen Koch for help with refolding of PopC proteins. The German Research Council within the framework of the Collaborative Research Centre 395 "Interaction, Adaptation and Catalytic Capability of Soil Microorganisms" and the Max Planck Society supported this work.

Received: March 24, 2008
Revised: July 2, 2008
Accepted: August 8, 2008
Published: October 13, 2008

**REFERENCES**


