Signal Recognition Particle (SRP)-mediated Targeting and Sec-dependent Translocation of an Extracellular Escherichia coli Protein*

Received for publication, November 14, 2002, and in revised form, December 2, 2002 Published, JBC Papers in Press, December 3, 2002, DOI 10.1074/jbc.M211630200

Robert Sijbrandi‡, Malene L. Urbanus‡, Corinne M. ten Hagen-Jongman‡, Harris D. Bernstein‡, Bauke Oudega‡, Ben R. Otto‡, and Joen Luirink‡‡

From the ‡Department of Molecular Microbiology, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands and the §Genetics and Biochemistry Branch, NIDDK, National Institutes of Health, Bethesda, Maryland 20892-1810

Hemoglobin protease (Hbp) is a hemoglobin-degrading protein that is secreted by a human pathogenic Escherichia coli strain via the autotransporter mechanism. Little is known about the earliest steps in autotransporter secretion, i.e. the targeting to and translocation across the inner membrane. Here, we present evidence that Hbp interacts with the signal recognition particle (SRP) and the Sec-translocon early during biogenesis. Furthermore, Hbp requires a functional SRP targeting pathway and Sec-translocon for optimal translocation across the inner membrane. SecB is not required for targeting of Hbp but can compensate to some extent for the lack of SRP. Hbp is synthesized with an unusually long signal peptide that is remarkably conserved among a subset of autotransporters. We propose that these autotransporters preferentially use the co-translational SRP/Sec route to avoid adverse effects of the exposure of their mature domains in the cytoplasm.

Signal Recognition Particle (SRP)-mediated Targeting and Sec-dependent Translocation of an Extracellular Escherichia coli Protein

Hemoglobin protease (Hbp) is a hemoglobin-degrading protein that is secreted by a human pathogenic Escherichia coli strain (1) and contributes to the pathogenic synergy between E. coli and Bacteroides fragilis in intra-abdominal infections (2). It represents the first described member of the serine protease autotransporters of Enterobacteriaceae (SPATE) group of autotransporter proteins (3).

The key feature of an autotransporter is that it contains all the information for secretion in the precursor of the secreted protein itself (3). Autotransporters comprise three functional domains: 1) an N-terminal targeting domain; 2) a C-terminal translocation domain; and, in between these two, 3) the passenger domain that is the actual secreted moiety. The C-terminal domain is supposed to form a β-barrel structure in the outer membrane that may form an oligomeric channel around a cavity to allow the passage of the passenger domain (4). The N-terminal domain is thought to function as a signal peptide to mediate targeting to and translocation across the inner membrane.

Compared with other signal peptides in E. coli, the putative signal peptide of Hbp is unusually long (1) (Fig. 1). Analogously, several other autotransporters are predicted to have long signal peptides (3, 5). All these signal peptides display a conserved domain structure. The C terminus resembles a normal signal peptide with a basic N-terminal region, a hydrophobic core region, and a C-terminal consensus signal peptide cleavage site. The N terminus forms a conserved extension, the function of which is not known (Fig. 1).

Most periplasmic and outer membrane proteins synthesized with a cleavable signal peptide are translocated through the Sec-translocon. The core translocase consists of the integral membrane proteins (IMPs) SecY, SecE, and SecG, which constitute an oligomeric complex homologous to the Sec61 channel complex in the endoplasmic reticulum (6). The peripheral membrane ATPase SecA is unique to bacteria and catalyzes the actual polypeptide transfer through the translocase. Targeting to the Sec-translocon may occur after translation and often requires the cytosolic chaperone SecB.

The Sec-translocon is also used for the membrane insertion of most IMPs that are synthesized with uncleaved, relatively hydrophobic signal peptides (7). Targeting of IMPs to the Sec-translocon is not mediated by SecB but by the signal recognition particle (SRP) and its receptor, FtsY, in a co-translational mechanism that resembles targeting to the Sec61 complex in the endoplasmic reticulum (8).

Here we provide evidence that the long signal peptide of Hbp mediates targeting to the inner membrane via the SRP pathway. When the SRP pathway is compromised, SecB can prevent, to a certain extent, the mislocalization of pre-pro-Hbp. Subsequent translocation across the inner membrane involves the Sec-translocon. This is the first demonstration of the use of the co-translational SRP pathway for inner membrane targeting of an extracellular protein.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Media—** E. coli K12-strains and the plasmids used are listed in Table I. E. coli strains were routinely grown in Luria-Bertani (LB) medium (9). Strains MM152 and HDB52 were grown in M9 medium (9). If required, antibiotics were added to the culture medium.

**Reagents and Sera—** Restriction enzymes, the Expand long template PCR system and the Lumilight Western blotting substrate were obtained from Roche Molecular Biochemicals. All other chemicals were supplied by Sigma. Antiserum J40 raised against purified Hbp has been described previously (2). Antisera against β-lactamase, OmpA/OmpC, trigger factor (TF)/SecA, and SufB were gifts from J.-M. van Dijl, J.-W. de Gier, W. Wickner, and T. Palmer, respectively.

**Plasmid Construction—** For cross-linking of nascent Hbp, we constructed pC4MethssHbp, which encodes the 110 N-terminal amino acid residues of Hbp fused to a C-terminal 4× methionine tag and a three amino acid linker sequence to improve labeling efficiency. The construct...
Targeting and Translocation of the Autotransporter Hbp

FIG. 1. The signal peptide of Hbp has a conserved N-terminal extension. A schematic representation of the 52 amino acid-long signal peptide of Hbp is shown, indicating the probable signal peptidase cleavage site (arrow). The basic N-terminal (N), hydrophobic (H), and C-terminal (C) domains characteristic of typical signal peptides are indicated. M indicates the beginning of the mature region of Hbp. A comparison of the N-terminal extensions of several autotransporters possessing extended signal peptides is given together with a consensus sequence of the conserved domain.

was obtained by PCR using pHE12.6 as a template and the primers Hbp-EcoRI-for (5'-GGCCGATATCTAATAGAAGATTTTCTTTCCC-3' with the EcoRI restriction site in boldface) and Hbp-BamHI-rev (5'-GGCCGGATCCACGGGTCAAGGATGCTGAGCTG-3' with the BamHI restriction site in boldface). The PCR fragment was cloned EcoRI/BamHI into pCM490 (10). To construct plasmid pHB6.4-HbpΔss, the signal peptide coding region of hbp was removed from plasmid pHB6.4-Hbp using the Exsite PCR-based, site-directed mutagenesis kit (Stratagene). The plasmid pHB6.4-Hbp is derived from pHE12.6 (1). The primers used were Hbp-Nhel-for (5'-CTTGCGAATTCGCTAATAATGAGCCGATATCTAATAGAAGATTTTCTTTCCC-3' with the NheI restriction site in boldface) and Hbp-3'-rev (5'-GCGAAGATCATTGTTTCTTTCCC-3' with the BgII restriction site in boldface). Upon ligation of the linear DNA, the last three bases from each end constitute a HindIII restriction site. The resulting plasmid encodes the Hbp protein without its signal peptide but with six extra N-terminal amino acids (MKKSLK) and two altered amino acids (GT → AS) at the start of the mature Hbp region.

In Vitro Transcription, Translation, Targeting, and Cross-linking—Truncated mRNA was prepared as described previously (11) from HindIII linearized pCM490-Hbp. In vitro translation, targeting to inserted membrane vesicles (IMVs), cross-linking with DSS, and carbonate extraction of nascent Hbp were carried out as described (11). The samples were either analyzed directly by 15% SDS-PAGE or immunoprecipitated first using 4-fold the amount used for direct analysis.

Pulse-Chase Experiments—For Ffh depletion studies, strains HBDB51 and HBDB52 were grown overnight in M9 containing 0.1% casamino acids (Difco), 0.2% fructose, and 0.2% L-arabinose, washed in the same medium lacking L-arabinose, and diluted to an OD 660 of 0.004 in M9 medium. 3 mM NaN3 was added 3 min. To inhibit SecA functioning in MC4100, 3 mM NaN3 was added to induce FtsY-A449 expression, and growth was continued for 15 min. The pulse labeling for 1 min by the addition of 10 μCi/ml [35S]methionine and chased for various times by adding cold methionine (2 mg/ml). To stop the chase, cells were rapidly cooled in ice water and centrifuged at 4°C for 2 min at 8,000 × g. Supernatants were precipitated with trichloroacetic acid and subjected directly to 8–15% SDS-PAGE. Cell pellets were first lysed and subjected to immunoprecipitation using anti-Hbp and anti-OmpA serum essentially as described (12).

Western Blotting—For analysis of steady-state levels of Hbp, E. coli strains harboring an hbp expression plasmid were grown to an OD 660 of 0.5. Aliquots were removed from the cultures and centrifuged (1 min at 16,000 × g). The culture supernatants were trichloroacetic acid precipitated. Equivalent amounts of cells and supernatant were analyzed by Western blotting. Blots were developed by enhanced chemiluminescence using Lumi-light Western blotting substrate.

Sample Analysis—Radiolabeled proteins were visualized by phosphorimaging using a Amersham Biosciences PhosphoImager 473 and quantified using the ImageQuant quantification software from Amersham Biosciences. Chemiluminescent Western blots were analyzed using the Fluor-S MultiImager and Multianalyst software (Bio-Rad).

RESULTS

Hbp That Lacks Its Signal Peptide Is Not Secreted and Degraded in the Cytosol—Hbp is synthesized in a pre-pro form (148 kDa) (1). The N-terminal signal peptide is cleaved during passage of the pre-pro-Hbp through the inner membrane, leaving the pro-Hbp (142 kDa) in the periplasm. The C-terminal β-barrel domain is cleaved from the pro-Hbp at the outer membrane and mediates the transfer of the mature Hbp (111 kDa) into the culture medium. To analyze the role of the Hbp signal peptide, it was deleted from the pre-pro-Hbp, and the consequences for maturation and secretion were analyzed by Western blotting of cell samples and culture supernatants using Hbp-specific antibodies (Fig. 2A). As expected, deletion of the signal peptide (Δss) prevented secretion of mature Hbp into the medium. Only a small amount of pro-HbpΔss was detected in the cells. In contrast, most wild-type pre-pro-Hbp was processed to mature Hbp, which was either secreted or remained cell-associated as observed previously (1). In addition, some pre-pro- or pro-Hbp accumulated in the cells.

We next investigated the reason for the low expression level of pro-HbpΔss. It has been observed previously that secreted proteins and IMFs that fail to be translocated across or inserted into the inner membrane are prone to proteolytic degradation (13). To investigate this possibility, HbpΔss was expressed in strain HDB107, which lacks the major cytoplasmic proteases Lon and ClpQ (13). Pulse-chase labeling was employed to compare the stability of HbpΔss species in strain HDB107 and its isogenic parental strain HBDB97 (Fig. 2B). HbpΔss remained almost completely stable for at least 10 min in HDB107, whereas only limited amounts were detected in HBDB97 after a 10-min chase. In fact, relatively little HbpΔss was detected in HBDB97 even when samples were analyzed directly after pulse labeling.

These data indicated that Hbp requires its signal peptide for targeting to the inner membrane and, consequently, for secretion of mature Hbp. Mislocalized pro-Hbp is rapidly degraded by the cytoplasmic proteases Lon and/or ClpQ.

Nascent Hbp Interacts with SRP, Trigger Factor, SecA, and SecY in Vitro—To investigate the molecular interactions of the atypical Hbp signal peptide in the cytoplasm and the membrane, we used an in vitro cross-linking assay. A radiolabeled Hbp translation intermediate of 117 amino acid residues was generated by in vitro translation of truncated mRNA in a homologous cell-free translation system developed previously in our laboratory (11). Because the truncated RNA does not contain a stop codon, the nascent chain remains associated with the ribosome, and the signal peptide is exposed outside the ribosome that covers ~35 C-terminal amino acids. Translation was carried out in the presence of purified IMVs to allow targeting and membrane insertion. Subsequently, interactions of the nascent Hbp were fixed by using the membrane-permeable, lysine-specific cross-linking reagent DSS. Finally, the samples were extracted with sodium carbonate to separate membrane integrated from the soluble and peripheral membrane proteins.
HDB107 cells. pro

Top10F

was grown to an OD 660 of 0.5 and split in cells and supernatant (quantified amount of pro-Hbp. cell samples were immunoprecipitated using anti-Hbp serum and sub-

4656

cytosol. HDB97 (wild-type) and HDB107 (Lon and/or ClpYQ are involved in degradation of precursor Hbp in the

lane 4

rected against Ffh, the protein component of the SRP (Fig. 3, lane 4). The molecular mass of this product is consistent with

FIG.2 .

A

approximately 30% of the synthesized nascent Hbp was de-

B

lanes 1–7, and subsequently extracted with carbonate (supernatant (sup), lanes 1–4; pellet, lanes 5–7). DSS-treated fractions

were immunoprecipitated using anti-Hbp serum and sub-

FIG.3 .

were either treated with DSS (lanes 5 and 6) or mock treated (lanes 1 and 5) and subsequently extracted with carbonate (supernatant (sup), lanes 1–4; pellet, lanes 5–7). DSS-treated fractions

were immunoprecipitated using antiserum against TF, Ffh, and SecA (lanes 3, 4, and 7). The translation products at 30 kDa present in lanes 5 and 6 represent the peptidyl-tRNA form of nascent Hbp. Cross-linked products and nascent chains (NC) are indicated.

The

approximately 30% of the synthesized nascent Hbp was detected in the carbonate pellet (Fig. 3, quantification data not shown). Given the relatively low intrinsic efficiency of the E. coli in vitro translocation system, this result indicated that nascent Hbp is properly targeted and inserted into the membrane and remains anchored via its signal peptide that is not cleaved at this nascent chain length. In the untargeted (carbonate soluble) fraction, a cross-linking product of ~60 kDa appeared that could be immunoprecipitated using serum directed against Ffh, the protein component of the SRP (Fig. 3, lane 4). The molecular mass of this product is consistent with the combined molecular mass of Ffh (50 kDa) and the Hbp 117-mer (13 kDa). The cross-linking to Ffh is remarkably strong considering the low abundance of the SRP in the translation lysate, suggesting that it represents a functional interaction. Weaker cross-linking products of slightly higher molecular mass were immunoprecipitated using anti-TF serum (Fig. 3, lane 3). TF (54 kDa) cross-linked to nascent chains often

migrates at varying positions (10). The membrane-integrated nascent Hbp was primarily cross-linked to SecA (102 kDa) (Fig. 3, lane 7). In addition, a very faint cross-linking smear was
observed at ~46 kDa that contained SecY, as evident from long exposures of immunoprecipitated samples (not shown).

Together, the cross-link patterns are reminiscent of those found with the nascent IMPs FtsQ and leader peptidase I (Lep) (10, 11, 14). Both FtsQ and leader peptidase I are targeted by the SRP to the Sec-translocon (15, 16). Apparently, nascent Hbp can be targeted to the inner membrane and inserts close to the Sec-translocon. The unprecedented strong cross-linking to Ffh, considering the extremely low abundance of Ffh in the translation lysate, suggests a high affinity of the Hbp signal peptide for the SRP and, consequently, a role for the SRP in the targeting of Hbp.

**Hbp Requires the SRP for Optimal Processing and Secretion in Vivo**—We investigated whether the interaction of nascent Hbp with the SRP observed in vitro reflects a dependence on the SRP targeting pathway for processing and secretion of the full-length protein in vivo. Strains that are conditional for the expression of targeting factors were used in pulse-chase experiments to analyze the effects on the kinetics of processing as described above. Furthermore, spent medium of the pulse-chase samples was analyzed to monitor the secretion of mature Hbp in time.

Under normal conditions, N-terminal processing appeared to be very fast, and pre-pro-Hbp could only be detected in the pulsed sample (Fig. 4A, lane 7) as has been observed for many pre-secretory proteins such as OmpA (see also Fig. 4C, lane 4). However, C-terminal processing of Hbp is much slower. Under the expression conditions used, not all pro-Hbp was converted into mature Hbp even after 1 h of chase (Fig. 4A, lane 12). The actual release of mature Hbp into the culture medium is even slower, appearing prominently only after 1 h of chase (Fig. 4B, lane 12). Depletion of Ffh resulted in an accumulation of pre-pro-Hbp in the cells (Fig. 4A, left panel). The amount of secreted mature Hbp after 60 min of chase, but not the kinetics of the secretion of mature Hbp, appeared affected upon depletion of Ffh (Fig. 4B, left panel). As a control, the processing of OmpA was hardly influenced (Fig. 4C, left panel), which is in agreement with its requirement for SecB rather than SRP for tar-
getting (17). In an alternative approach to analyzing the role of the SRP-targeting pathway in Hbp secretion, the effect of overexpression of FtsY-A449 was investigated. This mutant SRP receptor has a reduced GTP-binding capacity as a result of an amino acid substitution in the fourth GTP-binding consensus element (18). Moderate overexpression of FtsY-A449 has been shown to compromise SRP-mediated protein targeting (18). Hbp processing and, consequently, also secretion appeared to be impaired upon moderate overexpression of FtsY-A449 as opposed to the non-induced expression level (Fig. 4, panels B and E). OmpA processing was not affected under these conditions (Fig. 4F).

To examine whether the other major targeting factor, SecB, is also involved in Hbp targeting, Hbp was expressed in strain MM152, which lacks SecB, and in its isogenic wild-type strain, MC4100. Pre-pro-Hbp did not accumulate in the SecB minus strain, nor was the secretion of mature Hbp affected (Fig. 4, left panel, A and C). Again, pre-pro-Hbp accumulated in the secY mutant strain (Fig. 5, left panel, A). This double mutant strain showed normal kinetics of OmpA processing and secretion of Hbp whereas processing of the TatE, rendering it completely unable to translocate Tat substrates. This double mutant strain showed normal kinetics of processing and secretion of Hbp whereas processing of the known Tat substrate SufI was completely blocked, arguing that the Tat-translocon is not involved in secretion of Hbp (data not shown).

The Tat-translocon is used by a subset of preproteins that are folded prior to translocation (19). Tat substrates carry an essential twin arginine motif in their signal peptide just upstream of the hydrophobic domain. Although the Hbp signal peptide does not fully comply with this motif, it does contain two consecutive arginine residues upstream of the hydrophobic core region. This feature prompted us to investigate a possible role of the Tat-translocon using a strain that lacks TatA and TatE, rendering it completely unable to translocate Tat substrates. This double mutant strain showed normal kinetics of processing and secretion of Hbp whereas processing of the known Tat substrate SufI was completely blocked, arguing that the Tat-translocon is not involved in secretion of Hbp (data not shown).

Together, the data suggest that Hbp uses the Sec-translocon for transfer across the inner membrane consistent with the in vitro cross-link data (Fig. 3). SecA appears to be required to drive the translocation process.

DISCUSSION
In the present work, we have addressed the question how the autotransporter Hbp is targeted to and translocated across the inner membrane. Both in vitro cross-linking and in vivo pulse-chase labeling experiments point to the use of a co-transla-
Targeting and Translocation of the Autotransporter Hbp

In addition, substrates of an analogous secretion system, the “two-partner secretion” (TPS) pathway in which the β-barrel domain is present in a separate protein, also possess signal peptides that are conserved with members of the classical autotransporter family (5). One of these substrates, the HMW1 adhesin from *Haemophilus influenzae* that carries a 68 amino acid-long signal peptide was shown to require SecA and SecE for maturation and secretion (25).

What would be the benefit of co-translational translocation for autotransporters? For Hbp, it might prevent degradation or premature folding of Hbp in the cytoplasm. Hbp that lacks its signal peptide appeared to be vulnerable to degradation by cytoplasmic proteases. It should be noted that both the autotransporter and two-partner secretion families comprise many virulence factors such as hemagglutinins, hemolysins, cytolsins, and proteases (5) that may be harmful when expressed in the cytoplasm of the pathogenic bacterium. Furthermore, the autotransporters are relatively large molecules with a typical domain structure. The passenger domain that is expressed in the cytoplasm may fold into a conformation that is incompatible with translocation through the Sec-translocon, even when the β-barrel domain is still being synthesized.

REFERENCES

1. Otto, B. R., van Dooren, S. J., Nuijens, J. H., Lurijink, J., and Oudega, B. (1998) *J. Exp. Biol.* 188, 1091–1103
2. Otto, B. R., van Dooren, S. J., Deuzis, C. M., Lurijink, J., and Oudega, B. (2002) *Infect. Immun.* 70, 5–10
3. Henderson, I. R., Navarro-Garcia, F., and Nataro, J. P. (1999) *Trends Microbiol.* 6, 370–378
4. Veiga, E., Sugawara, E., Nikaido, H., de Lorenzo, V., and Fernandez, L. A. (2002) *EMBO J.* 21, 2122–2131
5. Henderson, I. R., Cappello, R., and Nataro, J. P. (2000) *Trends Microbiol.* 8, 529–532
6. Manting, E. H., and Driessen, A. J. M. (2000) *Mol. Microbiol.* 37, 226–238
7. de Gier, J. W., and Lurijink, J. (2001) *Mol. Microbiol.* 40, 314–322
8. Herskovits, A. A., Bochkareva, E. S., and Bibi, E. (2000) *Mol. Microbiol.* 38, 927–939
9. Miller, J. H. (1992) *A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*, pp. 437–439, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
10. Valent, Q. A., de Gier, J. W. L., van Heijne, G., Kendall, D. A., ten Hagen-Jongman, C. M., Oudega, B., and Lurijink, J. (1997) *Mol. Microbiol.* 25, 53–64
11. Scotti, P. A., Urbanus, M. L., Brunner, J., de Gier, J. W., van Heijne, G., van der Does, C., Driessen, A. J. M., Oudega, B., and Lurijink, J. (2000) *EMBO J.* 19, 542–549
12. Lurijink, J., High, S., Hoom, H., Giner, A., Tellervrey, D., and Dobberstein, B. (1992) *Nature* 359, 741–743
13. Bernstein, R. D., and Hyndman, J. B. (2001) *J. Bacteriol.* 183, 2187–2197
14. Houben, E. N. G., Scotti, P. A., Valent, Q. A., Brunner, J., de Gier, J. W. L., Oudega, B., and Lurijink, J. (2000) *FEBS Lett.* 476, 229–233
15. Urbanus, M. L., Scotti, P. A., Froderberg, L., Saaf, A., de Gier, J. W. L., Brunner, J., Samuelsen, J. C., Dalbey, R. E., Oudega, B., and Lurijink, J. (2001) *EMBO Rep.* 2, 524–529
16. de Gier, J. W. L., Mansournia, P., Valent, Q. A., Phillips, G. J., Lurijink, J., and van Heijne, G. (1996) *FEBS Lett.* 389, 307–310
17. Kumasato, C. A., and Beckwith, J. (1985) *J. Bacteriol.* 163, 267–274
18. Kusters, R., Lentzen, G., Eppens, E., van Geel, A., van der Weijden, C. C., Wintemeyer, W., and Lurijink, J. (1995) *FEBS Lett.* 372, 253–258
19. Berks, B. C., Sargent, F., and Palmer, T. (2000) *Mol. Microbiol.* 35, 260–274
20. Kim, J., Rasch, S., Lurijink, J., and Kendall, D. A. (2001) *FEBS Lett.* 524, 245–248
21. Sarker, S. R., Ridd, K. E., and Oliver, D. (2000) *J. Bacteriol.* 182, 5592–5595
22. Nakagawara, H., and Ito, K. (2001) *Mol. Cell.* 7, 185–192
23. Batey, R. T., Rambo, R. P., Lucaet, L., Rha, B., and Doudna, J. A. (2000) *Science* 287, 1233–1239
24. Samuelsson, J. C., Chen, M. Y., Jiang, F. L., Moller, I., Wiedmann, M., Kuhn, A., Phillips, G. J., and Dalbey, R. E. (2000) *Nature* 406, 637–641
25. Gras, S., and St. Ghez, J. W., III (2000) *Mol. Microbiol.* 36, 55–67
26. Casadaban, M. J. (1976) *J. Mol. Biol.* 104, 541–555
27. Studier, F. W., Rosenberg, R. A., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol.* 185, 60–89
28. Lee, H. C., and Bernstein, H. D. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 3471–3476
29. Shibah, K., Ito, K., Yura, T., and Ceretti, D. P. (1984) *Science* 226, 532
30. Sargent, F., Stanley, N. R., Berks, B. C., and Palmer, T. (1999) *J. Biol. Chem.* 274, 36073–36082
31. van Dooren, S. J., Tame, J. R., Lurijink, J., Oudega, B., and Otto, B. R. (2001) *FEBS Microbiol. Lett.* 205, 147–150

2 E. N. G. Houben, personal communication.