JOURNAL OF BACTERIOLOGY, Oct. 2005, p. 6852–6855 Vol. 187, No. 19
0021-9193/05/$08.00+0 doi:10.1128/JB.187.19.6852–6855.2005

Inactivation of Protein Translocation by Cold-Sensitive Mutations in the *yajC-secDF* Operon

Nico Nouwen* and Arnold J. M. Driessen

Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

Received 28 April 2005/Accepted 25 July 2005

Most mutations in the *yajC-secDF* operon identified via genetic screens confer a cold-sensitive growth phenotype. Here we report that two of these mutations confer this cold-sensitive phenotype by inactivating the SecDF-YajC complex in protein translocation.

In bacteria, the Sec system provides the general route by which proteins are transported across or inserted into the cytoplasmic membrane. In *Escherichia coli*, the Sec system is composed of eight different proteins. The integral membrane proteins SecY, SecE, and SecG (SecYEG) constitute a heterotrimeric protein conducting channel complex to which the peripherally bound motor protein, the SecA ATPase, associates to form the core of the translocation machinery. The integral membrane proteins SecD, SecF, and YajC (SecDF-YajC) are organized in a distinct complex that can associate with the SecYEG complex (5). Biochemical analysis of the protein translocation reaction in vitro and structural analysis of components of the translocation machinery now provide considerable insight in the mechanistic role of the core components of the Sec machinery in protein translocation (for a review, see reference 3). However, the mechanistic role of the SecDF-YajC complex in protein translocation is far from understood. In vivo and in vitro studies on the role of SecD and SecF in protein translocation have yielded apparently conflicting results. Whereas cells lacking SecD and/or SecF have a severe export defect and are barely viable (10), in vitro protein translocation reconstituted with SecYEG proteoliposomes does not require SecDF-YajC (1, 2). SecD and SecF possess a very large first periplasmic domain that is important for catalyzing protein translocation (8). The genes encoding SecD, SecF, and YajC are localized in one operon (*yajC-secD-secF*) (12). Remarkably, most *secD* and *secF* mutants isolated via genetic screens are cold sensitive (Cs) for growth and exhibit translocation defects at both high and low temperatures (6, 7). Cs mutants might be helpful to reveal the mechanistic role of SecD and SecF in protein translocation. However, these mutations have never been mapped and it is generally thought that the cold-sensitive mutations in SecD and SecF confer this phenotype by a reduction in protein level (10, 11). Here we report on the characterization of two of the most often used cold-sensitive SecD and SecF mutants (SecD29 and SecF62) and demonstrate that the mutations directly inactivate the SecDF-YajC complex.

* Corresponding author. Mailing address: Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands. Phone: 31-60-3632403. Fax: 31-50-3632154. E-mail: n.p.nouwen@rug.nl.

In vivo analysis of *secD29* and *secF62* mutants. *E. coli* strains KJ173 (*secD29*) and KJ184 (*secF62*) were obtained from the *E. coli* Genetic Stock Center (New Haven, Conn.). Together with the parental wild-type strain MC1000, KJ173 and KJ184 mutant strains were plated onto Luria-Bertani (LB) plates and incubated at 37°C and 22°C, respectively. In agreement with published data, all three strains grow normally at 37°C, whereas strains KJ184 (*secF62*) and KJ173 (*secD29*) do not grow or grow poorly at 22°C (data not shown). To analyze the SecD and SecF levels in the different strains, cells were grown in LB broth at 37°C and inner membrane vesicles (IMVs) were isolated. Immunoblot analysis showed that in membranes from KJ184 (*secF62*) cells, the SecF level is lower than that in membranes from wild-type cells (Fig. 1A, lane 3 versus lane 1). In contrast, membranes from KJ173 (*secD29*) cells did not show a detectable difference in the SecD and SecF levels compared to membranes from the wild-type strain (Fig. 1A, lane 2 versus lane 1). However, in membranes of both KJ184 (*secF62*) and KJ173 (*secD29*) cells, the amount of membrane-associated SecA increased up to twofold compared to wild-type membranes (Fig. 1A, lanes 2 and 3 versus lane 1). Previously, it has been suggested that protein translocation includes a step that is intrinsically cold sensitive (11). A reduction in the level of one of the components of the translocation machinery readily results in a cold-sensitive growth phenotype. The strongly reduced amount of SecF62 in the membrane of KJ184 cells might therefore explain the Cs phenotype of this strain. However, in KJ173 cells, the levels of SecD29 and SecF are normal, and this strain also displays a Cs growth phenotype. For this reason, we decided to clone the *secD29* and *secF62* genes and to map the mutation that is responsible for the cold-sensitive phenotype.

Mapping of the mutations in the *secD29* and *secF62* genes. The *secD29* and *secF62* genes were amplified from genomic DNA of *E. coli* strains KJ173 and KJ184, respectively, by using the following primer pair sets: *secD29* (5′-GCTGCCGTCCTG CAGAAAGGCACCATGAAGGCGC-3′ and 5′-CCCAGCG CATAAAGTCATAAGCTTTACGGCCGTGG-3′) and *secF62* (5′-CCTGTACGCAGTGGGTACCGGGG-3′ and 5′-CAAC ATCAACGGGAAGCTTGATTACGGCAG-3′). The PCR products were cloned into pUC19, sequenced, and compared with the wild-type *secD* and *secF* sequences for the presence of nucleotide changes. The *secD29* gene of strain KJ173 contained a single nucleotide change (C to T) which results in a

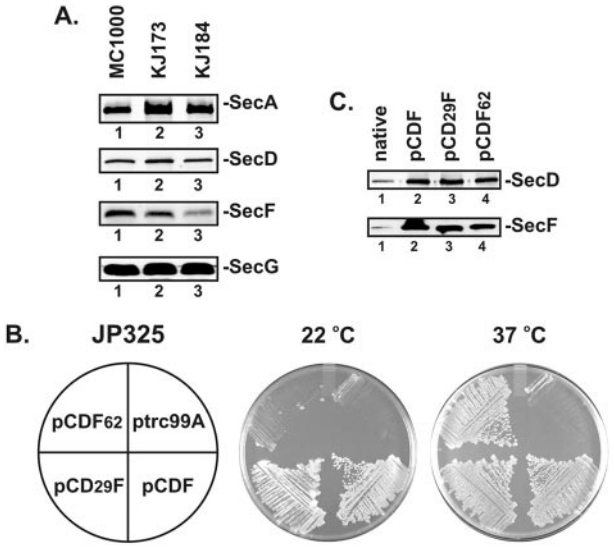

FIG. 1. (A) Inner membrane vesicles from the cold-sensitive *E. coli* strain KJ184 (*secF62*) contain a reduced level of SecF. The amounts of SecA, SecD, SecF, and SecG in inner membrane vesicles (5 to 20 μg protein) derived from *E. coli* strains MC1000 (wild type), KJ173 (*secD29*), and KJ184 (*secF62*) grown in LB broth at 37°C were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunostaining using antibodies against SecA, SecD, SecF, and SecG. (B) *E. coli* JP325 cells (P_{BAD}::*yajC-secDF*) transformed with either pCDF (pET545), pCD29F (pET599), pCDF62 (pET598), or an empty vector (ptrc99A) were plated onto LB-ampicillin (100 μg/ml) plates containing 0.5% glucose and incubated at 37°C (1 day) or 22°C (3 days). (C) IMVs from cells from which chromosome-encoded SecDF-YajC was depleted and which expressed SecD29F-YajC and SecDF62-YajC were prepared from strain JP325 containing plasmids pET599 (*yajC-secD29-secF*) and pET598 (*yajC-secD-secF62*), respectively. Cells were grown for six generations on LB medium containing 0.5% glucose, after expression of plasmid-encoded proteins had been induced with 100 μM IPTG. IMVs (5 to 10 μg protein) were analyzed by SDS-PAGE and immunostaining using antibodies against SecD and SecF and compared with the amount of SecD and SecF present in membranes of the wild-type *E. coli* strain SF100 (lane 1).

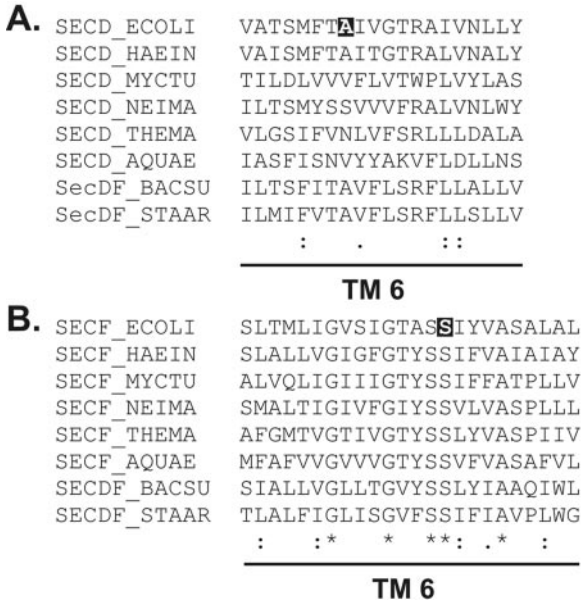

FIG. 2. Alignment of the last transmembrane segment of SecD and SecF proteins from different bacteria. (A) The SecD(F) proteins from *Escherichia coli*, *Haemophilus influenzae*, *Mycobacterium tuberculosis*, *Neisseria meningitidis* (serogroup A), *Thermotoga maritima*, "*Aquifex aeolicus*," *Bacillus subtilis*, and *Staphylococcus aureus* (strain MRSA252) were aligned by using the Clustal W program, and the last transmembrane segment of SecD containing the SecD29 mutation (A593V) is indicated. (B) The Sec(D)F proteins from the bacteria indicated for panel A were aligned by using the Clustal W program, and the last transmembrane segment of SecF containing the SecF62 mutation (S288F) is shown. Note the very-well-conserved double serine motif within the last transmembrane segment of SecF. Asterisk, identical amino acid; colon, strongly similar replacement; period, weakly similar replacement; TM 6, transmembrane segment 6. Positions of the Cs mutations are indicated in black.

replacement of the alanine residue at position 593 with a valine in the last transmembrane segment of SecD. This alanine residue is not conserved in the family of SecD proteins (Fig. 2A). Since valine residues are regularly present in transmembrane segments, the nature of the amino acid change does not readily explain why this substitution results in the Cs phenotype. In contrast, the nucleotide change in *secF62* (C to T) of strain KJ184 results in the replacement of the serine residue at position 288 with a phenylalanine. This serine residue is part of the very-well-conserved double serine motif present in the last transmembrane segment of SecF (Fig. 2B). The latter may explain the drastic effect of the mutation on SecF.

Functional expression of SecD29F-YajC and SecDF62-YajC. To investigate if the identified amino acid substitutions are indeed responsible for the Cs growth phenotype, we subcloned the two mutations into plasmid pET545. In this plasmid, the *yajC-secDF* operon is under control of the *trc* promoter and SecF contains a C-terminal six-histidine tag. Like the parental strains KJ184 and KJ173, strain JP325 (chromosome-encoded *yajC-secD-secF* operon under the control of the arabinose promoter) with plasmid pCDF62 or pCD29F grows normally at 37°C. However, this strain does not grow (JP325 and pCDF62) or grows poorly and forms mucoid colonies (JP325 and pCD29F) at 22°C when chromosome-encoded SecDF-YajC is switched off by growth in glucose-containing medium (Fig. 1B). To investigate if the Cs growth phenotype can be rescued by an increase in the amount of SecDF-YajC complex in the membrane, we induced the expression of the plasmid-encoded SecDF-YajC by addition of a low concentration of IPTG (isopropyl-β-D-thiogalactoside; 100 μM) to the growth medium. Immunoblot analysis showed that under these conditions, the amount of plasmid-encoded SecD and SecF in the membrane is about 10-fold higher than that of a wild-type *E. coli* strain (Fig. 1C, lanes 2 through 4 versus lane 1). However, also under these conditions, plasmids pCDF62 and pCD29F were unable to restore growth of the SecDF-YajC-depleted strain. In addition, high overproduction of the mutant SecDF-YajC complexes in a wild-type *E. coli* strain (MC4100) results in a dominant-negative growth phenotype (data not shown). These results demonstrate that the identified point mutations are responsible for the Cs growth phenotype and indicate that the

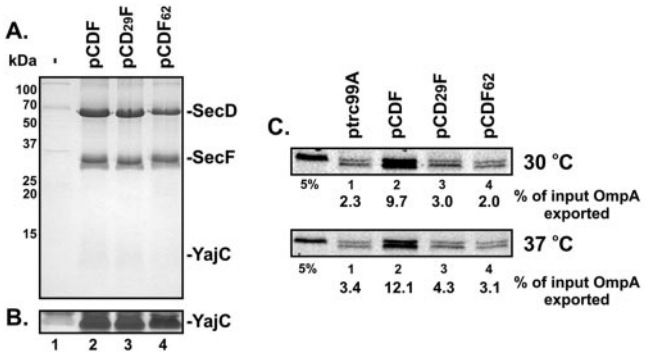

FIG. 3. SecD29 and SecF62 form a stable SecDF-YajC complex. IMVs were prepared from *E. coli* JP325 cells from which the chromosome-encoded SecDF-YajC was depleted and which overproduced SecD29 and SecF62 mutant SecDF-YajC. After solubilization of the IMVs with dodecylmaltoside, the mutant SecDF-YajC complexes were purified with Ni^{2+}-nitrilotriacetic acid beads and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue (A) or silver staining (B). Since JP325 cells with the Cs mutant plasmids or control plasmid do not grow at low temperature, IMVs are isolated from cells grown at 37°C. Purification of SecDF-YajC complexes has been performed at 4°C. IMVs containing SecD29F-YajC and SecDF62-YajC are defective in in vitro translocation. Translocation of pro-OmpA–fluorescein (4 μg/ml) into IMVs (0.2 mg/ml) from cells from which chromosome-encoded SecDF-YajC was depleted and which expressed wild-type, SecD29, or SecF62 SecDF-YajC from a plasmid was performed in 50 mM HEPES-KOH, pH 7.9, 50 mM KCl, 5 mM $MgCl_2$, 0.2 mg/ml bovine serum albumin, 2 mM dithiothreitol supplemented with SecA (10 μg/ml), SecB (35 μg/ml), 0.5 mM ATP, 10 mM phosphocreatine, and 50 μg/ml creatine kinase, 5 mM succinate, and 10 mM NADH. After 10 (37°C) or 15 (30°C) minutes at the indicated temperature, the reaction was stopped by the addition of proteinase K (0.1 mg/ml). (C) Translocation was assayed as described in reference 4. 5%, 5% standard of total added pro-OmpA.

primary cause of this phenotype is due to a defect in the mutant SecDF-YajC complex.

SecD29 and SecF62 form a stable SecDF-YajC complex. SecD, SecF, and YajC form a stable heterotrimeric complex in detergent solution (5). To determine if the SecD29 and SecF62 mutations affect complex formation, IMVs from JP325 cells in which chromosome-encoded SecDF-YajC has been depleted and which overproduce the SecD29 and SecF62 mutant were solubilized with the detergent dodecylmaltoside. Upon incubation of the membrane extract with Ni^{2+}-nitrilotriacetic acid agarose beads, both SecD and YajC copurify with the histidine-tagged SecF (9) (Fig. 3A and B, lane 2). The same copurification occurs with the membrane extract from cells overproducing SecD29 and SecF62 (Fig. 3A and B, lanes 2 and 3). This demonstrates that the point mutations do not affect complex formation between subunits of the SecDF-YajC complex.

In vitro translocation into inner membrane vesicles from cells expressing SecD29F-YajC and SecDF62-YajC is impaired. In vitro translocation of fluorescein-labeled pro-OmpA into IMVs from cells in which SecDF-YajC has been depleted is very inefficient (Fig. 3C, lane 1). However, pro-OmpA translocation can be restored to wild-type levels when the cells from which chromosome-encoded SecDF-YajC has been depleted (over)express wild-type SecDF-YajC from a plasmid (Fig. 3C, lane 2 versus lane 1). In contrast, when IMVs are used from cells bearing a plasmid that contains a *yajC-secDF* operon with the Cs *secD29* and *secF62* alleles, pro-OmpA translocation is similar to translocation into SecDF-YajC-depleted IMVs (Fig. 3C, lanes 3 and 4 versus lane 1). As under the tested conditions, the amounts of SecD and SecF in the membrane are in excess of the natural levels, and the mutant proteins form a stable SecDF-YajC complex (Fig. 3A and B); the results demonstrate that both the SecD29 and SecF62 mutations result in a nonfunctional protein.

The Cs growth phenotypes of some mutations in Sec components have been shown to be the result of a reduced expression level of the respective component(s) of the translocation machinery (13). Our study demonstrates that also at increased expression levels, Cs mutations in SecD and SecF result in a Cs growth phenotype. However, as protein translocation is sensitive to cold (11), mutations that affect but do not completely block the function of the translocation machinery will give a similar phenotype. The *E. coli* strains investigated in this paper and containing the Cs mutations show a translocation defect directly after the shift to the restrictive temperature (11). This instant (and reversible) translocation defect suggests that the activity of existing SecD and SecF molecules is impaired at low temperatures. Here we demonstrate that in membranes from cells grown at the permissive temperature, the two Cs SecD and SecF mutants form a stable SecDF-YajC complex. As the purification of the (mutant) SecDF-YajC complexes has been performed at a low temperature (4°C), the data indicate that the Cs phenotype of the SecD and SecF mutation is not the result of cold-induced dissociation of the mutant SecDF-YajC complex. Taken together, our data show that the two characterized Cs mutations yield an inactive SecDF-YajC complex, which results in an impaired in vitro protein translocation. Since genetic screens have identified many Cs mutations in the *yajC-secDF* operon (7), it would be of interest to map and analyze them with the procedure described here. In combination with biochemical studies, this may help to clarify the mechanistic role of the SecDF-YajC complex in protein translocation.

We thank G. Berrelkamp for expert technical assistance and V. Robert for critically reading the manuscript.

This work was supported by the Royal Academy of Sciences of The Netherlands (KNAW).

REFERENCES

1. **Akimaru, J., S. Matsuyama, H. Tokuda, and S. Mizushima.** 1991. Reconstitution of a protein translocation system containing purified SecY, SecE, and SecA from *Escherichia coli*. Proc. Natl. Acad. Sci. USA **88:**6545–6549.
2. **Brundage, L., J. P. Hendrick, E. Schiebel, A. J. M. Driessen, and W. Wickner.** 1990. The purified *E. coli* integral membrane protein SecY/E is sufficient for reconstitution of SecA-dependent precursor protein translocation. Cell **62:**649–657.
3. **de Keyzer, J., C. van der Does, and A. J. M. Driessen.** 2003. The bacterial translocase: a dynamic protein channel complex. Cell. Mol. Life Sci. **60:** 2034–2052.
4. **de Keyzer, J., C. van der Does, and A. J. M. Driessen.** 2002. Kinetic analysis of the translocation of fluorescent precursor proteins into *Escherichia coli* membrane vesicles. J. Biol. Chem. **277:**46059–46065.
5. **Duong, F., and W. Wickner.** 1997. Distinct catalytic roles of the SecYE, SecG and SecDFyajC subunits of preprotein translocase holoenzyme. EMBO J. **16:**2756–2768.
6. **Gardel, C., S. Benson, J. Hunt, S. Michaelis, and J. Beckwith.** 1987. *secD*, a new gene involved in protein export in *Escherichia coli*. J. Bacteriol. **169:** 1286–1290.
7. **Gardel, C., K. Johnson, A. Jacq, and J. Beckwith.** 1990. The *secD* locus of *E. coli* codes for two membrane proteins required for protein export. EMBO J. **9:**3209–3216.

8. **Nouwen, N., M. Piwowarek. G. Berrelkamp., and A. J. M. Driessen.** 2005. The large first periplasmic loop of SecD and SecF plays an important role in SecDF functioning. J. Bacteriol. **187:**5857–5860.
9. **Nouwen, N., and A. J. M. Driessen.** 2002. SecDFyajC forms a heterotetrameric complex with YidC. Mol. Microbiol. **44:**1397–1405.
10. **Pogliano, J. A., and J. Beckwith.** 1994. SecD and SecF facilitate protein export in *Escherichia coli*. EMBO J. **13:**554–561.
11. **Pogliano, K. J., and J. Beckwith.** 1993. The Cs *sec* mutants of *Escherichia coli* reflect the cold sensitivity of protein export itself. Genetics **133:**763–773.
12. **Pogliano, K. J., and J. Beckwith.** 1994. Genetic and molecular characterization of the *Escherichia coli secD* operon and its products. J. Bacteriol. **176:** 804–814.
13. **Schatz, P. J., K. L. Bieker, K. M. Ottemann, T. J. Silhavy, and J. Beckwith.** 1991. One of three transmembrane stretches is sufficient for the functioning of the SecE protein, a membrane component of the *E. coli* secretion machinery. EMBO J. **10:**1749–1757.