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ACS chemical biology

Intein-Mediated Cyclization of Randomized Peptides in the Periplasm of *Escherichia coli* and Their Extracellular Secretion

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ABSTRACT Split-inteins can be used to generate backbone cyclized peptide as a source of new bioactive molecules. In this work we show that cysteine-mediated splicing can be performed in the oxidative environment of the periplasm of *Escherichia coli*. Cyclization of the TEM-1 β-lactamase and of small randomized peptides was demonstrated using an artificially permuted version of the DnaB mini-intein from *Synechocystis* sp. PCC6803 strain fused to a signal sequence. For small peptides, a signal sequence that promotes cotranslational translocation had to be used. Efficient backbone cyclization was observed for more than 50% of combinatorial peptides featuring a fully randomized sequence inserted between a serine and glycine that are necessary for fast splicing. Furthermore, by coexpressing a mutant of the pIV outer membrane pore protein of fd bacteriophage, we showed that peptides can diffuse in the extracellular medium. These results open new routes for searching compounds acting on new targets such as exported and membrane proteins or pathogen microorganisms.

By providing large and diverse libraries of small molecules, chemical and biological methods offer an opportunity for developing potentially useful therapeutics (*1*–*3*). Libraries of small synthetic molecules can be rich in chemical diversity but often necessitate laborious screening assays. On the other hand, very complex biosynthetic compounds can be produced by living organisms and the genotype-phenotype linkage makes these systems amenable to high-throughput selective approaches.

In this context, the biosynthesis of "non-natural" natural products is a very active area of research. One of the most impressive success stories is the genetic engineering of polyketide and nonribosomal polypeptide synthases (PKS and NRPS), which has already led to the biosynthesis of several hundreds of new compounds (*4*–*6*). However, since these compounds are biosynthesized by specific and complex multienzymatic systems, high-throughput combinatorial approaches cannot be easily developed. In this regard, the SICLOPPS technology is an interesting alternative since it allows the biosynthesis of backbone cyclic peptides from ribosomally synthesized precursors (*7*, *8*). SICLOPPS is based on intein fragmentation and arrangement of the two fragments Int_N (the N-terminal part) and Int_C (the C-terminal part) in the permutated order Int_C-target sequence-Int_N. In this arrangement, the autocatalytic splicing reaction results in cyclization of the target sequence (*9*). The SICLOPPS technology has been used to produce cyclic peptides libraries of up to 10^8 members and has proven large flexibility toward peptide length and composition (*10*). Cyclic peptides libraries have been combined with different strategies for the genetic selection of active

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Received for review March 23, 2010 and accepted June 8, 2010.

Published online June 8, 2010

10.1021/cb100072u

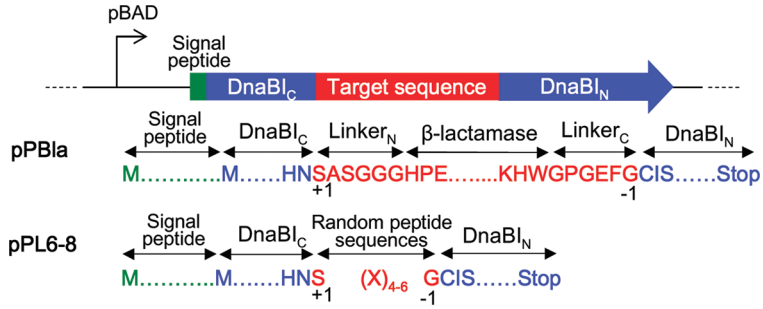

Figure 1. Constructs for protein and peptide cyclizations in the periplasm of *E. coli*. The target sequence for cyclization is cloned in frame between genes encoding the two fragments of the Ssp DnaB split intein (DnaBI$_C$ and DnaBI$_N$) in a permuted order and behind the arabinose controlled pBAD promoter. The pPBla construct illustrates the intein-TEM-1 β-lactamase precursor. Splicing will occur in the linker region connecting the N- and C-termini of the enzyme and is made possible by imposing a serine (+1) and a glycine (−1) at the splicing junctions. The pPL6-8 constructs for the cyclization of hexa-, hepta-, and octa-peptides libraries in the periplasm of *E. coli*. The randomized codons encoding four, five, or six amino acids are cloned between the fixed codons of the serine and glycine residues. The degeneracy of the codons is NNB (B = C, T, or G).

compounds. Using a bacterial reverse two-hybrid system (RTHS), a strategy for selecting cyclic peptides that modulate protein–protein interactions has been developed (*11, 12*). This strategy has been validated for the disruption of the ATIC homodimer (*13*) and for the inhibition of the Gag-TSG101 interaction that prevent the HIV budding event (*14*). In combination with, respectively, a fluorescent reporter substrate or an *in vivo* selectable transposase activity, cyclic peptide inhibitors of, respectively, the ClpXP protease (*15*) and the EcoDam DNA methyltransferase (*16*) were identified in *Escherichia coli.* Libraries of random cyclic peptides were also produced in mammalian cells with retroviral technology and a genetic screen has been set up to test their functionality (*17*).

With the aim of expanding the scope of applications of cyclic peptide libraries, we have evaluated the possibility to perform split intein-mediated polypeptide cyclization in the periplasm of *Escherichia coli* and further secrete the peptides in the extracellular medium. First, we demonstrated that a wide variety of intein-polypeptide precursors can be translocated in the periplasm prior to splicing. Second, we showed that minimal sequence determinants in the polypeptide precursors are sufficient for cyclization to occur prior to intein inactivation by oxidation of its essential cysteine in the periplasm. Various libraries of randomized precursors were created, and a large proportion of the clones were shown to efficiently produce backbone cyclic peptides in the periplasm. Finally, by expressing a phage pore protein in the outer membrane of *E. coli,* we showed that cyclic peptides could diffuse in the extracellular medium. These new possibilities of producing libraries of cyclic peptides either in the periplasm or outside the bacteria opens a wide potential of development of selection-based strategies for identifying compounds that would act on exported or membrane proteins or would interfere with competing organisms.

RESULTS AND DISCUSSION

Intein Splicing in the Periplasm. We initially decided to work with an artificially permuted mini-intein derived from the DnaB intein of *Synechocystis* sp. PCC6803 strain (called Ssp DnaB) because it appears to be well appropriate for producing high levels of cyclized target proteins (*18*). The TEM-1 β-lactamase (Bla) was chosen as the model periplasmic protein since its activity and stability in the cyclic form has been already demonstrated in the cytoplasm (*19*) and because its localization in the periplasm can be easily demonstrated by ampicillin resistance phenotype.

On the basis of the work of Liu *et al.* (*20*), the centrally located endonuclease domain of the Ssp DnaB intein was deleted to create a mini-intein of 154 aa split into two parts, a 106 aa N-terminal fragment (DnaBI$_N$) and a 48 aa C-terminal fragment (DnaBI$_C$). The two fragments were arranged in the order DnaBI$_C$-DnaBI$_N$ and cloned into an expression vector behind the arabinose-controlled pBAD promoter (Figure 1). To avoid a potential problem of oxidation in the periplasmic space, the nonessential Cys50 of the DnaBI$_N$ gene was mutated to alanine. The gene encoding Bla was then introduced between the two fragments by fixing a serine and a glycine codon as the first extein residue at the N-splicing junction and the last extein residue at the C-splicing junction (position +1 and −1 in Figure 1). Indeed, the serine is an essential residue involved in the splicing reaction (*21*) and the glycine was reported to be impor-

tant for efficient splicing of the Ssp DnaB mini-intein (*22, 23*). Sequences encoding five additional amino acids were also added on both sides of the Bla sequence providing a linker sequence of 12 residues that will connect the original N- and C-termini of the protein as in the previously reported cytoplasmic construct (*19*). Finally, a sequence encoding the signal peptide of Bla was fused at the 5′ side of the ORF (Figure 1, pPBla vector). In this construct, the splicing reaction must occur after export for the enzyme to be localized in the periplasm. An ORF without the signal sequence was also built and used as a negative control (pCBla vector).

In contrast to the pCBla transformants, bacteria transformed by the pPBla vector were fully resistant to high ampicillin concentration (100 $\mu g\ mL^{-1}$), therefore indicating a periplasmic localization. Moreover, SDS–PAGE analysis of the cytoplasmic and periplasmic extracts derived from induced cells for both constructions clearly confirmed the signal peptide dependent localization of Bla (Figure 2, panel a, compare lanes 3 *vs* 7 and lanes 9 *vs* 5). The band correponding to Bla migrated at around 30-kDa indicating that the intein has been spliced out (MW = 29849.9 Da for the circularized enzyme). Two other bands corresponding to the intein fragments $DnaBI_N$ and $DnaBI_C$ (11985 and 5544 Da) are also observed at about 12 and 5.5 kDa only under conditions of induction. Mass spectrometry analysis confirmed that those bands were indeed intein fragments. Importantly, no other byproducts possibly coming from inefficient or partial splicing reaction were detected. To prove the cyclization of Bla, the enzyme was purified from the periplasmic extract and analyzed by mass spectrometry after trypsin digestion. A 2107.2 Da peptide corresponding to the expected sequence resulting from the condensation of the N- and C-termini was clearly observed while intein peptides and junction peptides coming from either unspliced or cleaved products were not (Supplementary Figure 1, panel a).

Interestingly, with the pPBla periplasmic construct, the β-lactamase appears completely absent from the cytoplasm upon induction (Figure 2, panel a, compare lanes 6 *vs* 7). Since the signal peptide is fused to the intein, this indicates that there is no splicing prior to exportation.

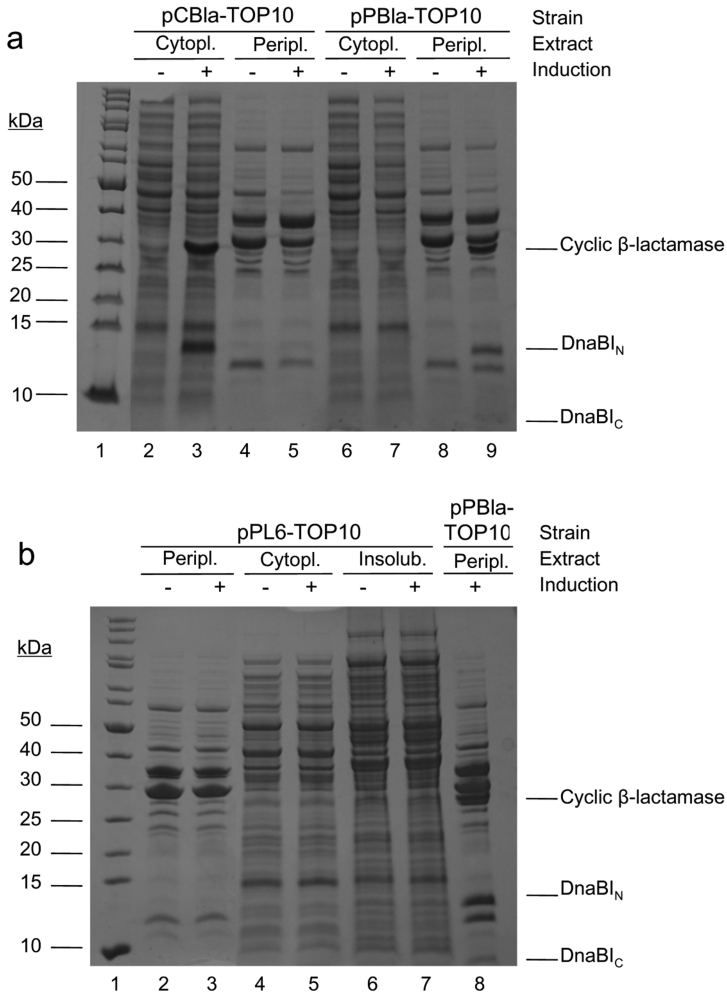

Figure 2. SDS–PAGE analysis of cytoplasmic and periplasmic extracts. a) *E. coli* TOP10 transformed with the vectors designed for the expression of cyclic β-lactamase in the cytoplasm (pCBla) or periplasm (pPBla) and under inducing (arabinose) or non-inducing conditions have been analyzed. Upon arabinose induction, bands corresponding to the cyclic β-lactamase and intein fragments are observed in the expected extracts. Lane 1: molecular weight ladder. b) Insoluble fraction and cytoplasmic and periplasmic extracts of *E. coli* TOP10 transformed with the periplasmic expression library of randomized cyclic hexapeptides (pPL6) under inducing (arabinose) or non-inducing conditions have been analyzed by SDS–PAGE. Intein fragments or precursors are not observed in any of the samples. The periplasmic extract obtained from the clone expressing the periplasmic cyclic β-lactamase is shown as a control (lane 8). Lane 1: molecular weight ladder.

Cyclic Peptides in the Periplasm. On the basis of this demonstration of intein functionality in the periplasm, the cyclic peptide libraries (pPL6-8) were similarly constructed by introducing, in place of the Bla gene, codons for four, five, or six amino acids between the codons encoding the serine and glycine residues ($SX_{(4\text{-}6)}G$, Figure 1). A NNB codon degeneracy (B = C, T, or G) was chosen, affording 48 codons that encode the

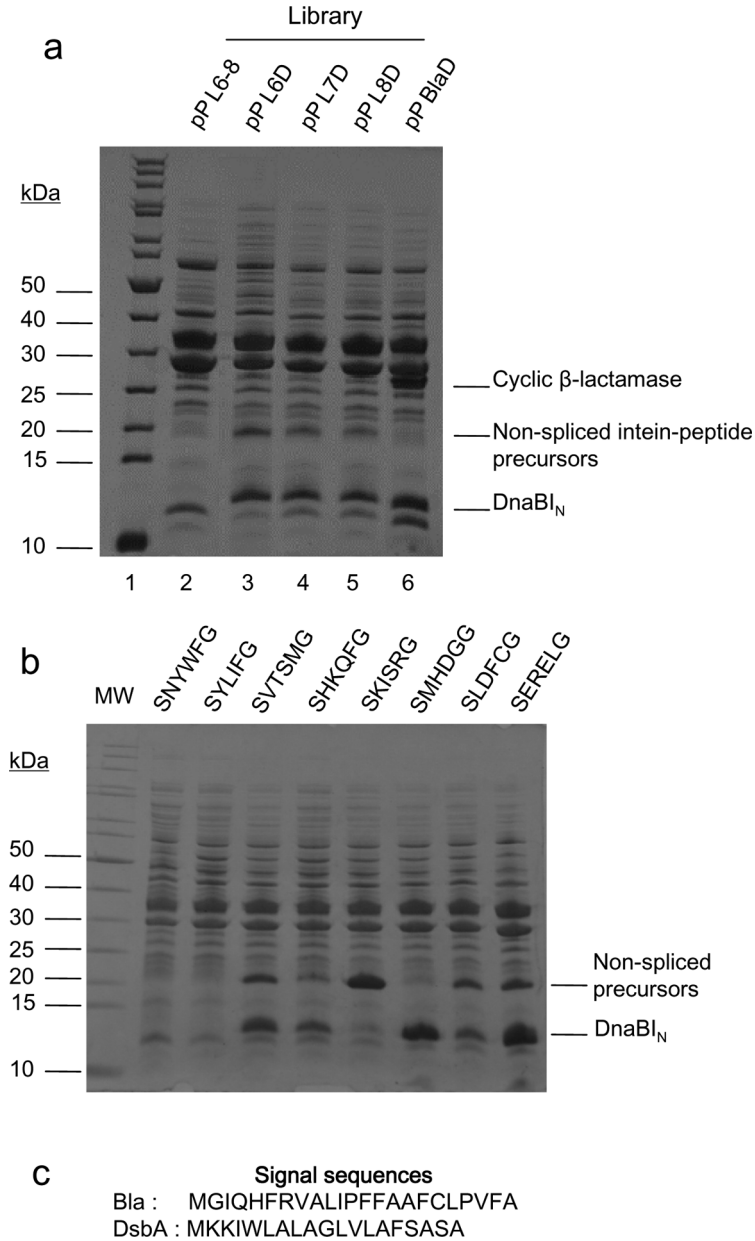

Figure 3. Precursors' export requires an SRP-dependent signal peptide. a) Comparison of the periplasmic extracts obtained from the arabinose-induced libraries expressing the precursors fused to the β-lactamase (lane 2) or DsbA signal peptide (lanes 3–5). In lane 2, the libraries encoding randomized hexa-, hepta-, and octa-peptides have been mixed. For the constructs with DsbA signal, each library (pPL6D, pPL7D, and pPL8D) was analyzed separately. Bands corresponding to the DnaBI$_N$ fragment and unspliced precursors are observed when the DsbA signal is used. The periplasmic extract obtained from the clone expressing the β-lactamase precursor with DsbA signal peptide is shown as a control (lane 6). Lane 1: molecular weight ladder. b) SDS–PAGE analysis of periplasmic extracts prepared from arabinose-induced cultures of eight clones picked at random in the hexa-peptide library pPL6D. The sequences of the encoded peptides are shown on the top of the gel. Depending on the clone, the DnaBI$_N$ fragment and the unspliced precursor are or are not observed. c) Sequences of Bla and DsbA signal peptides.

20 amino acids and only 1 stop. In the SDS–PAGE analysis of periplasmic extracts derived from the induced hexa-peptide library, the two intein fragments released after the splicing were not observed (Figure 2, panel b, lane 3) contrary to the β-lactamase result. Moreover, these bands were not present in the cytoplasmic extract or in the insoluble fraction (Figure 2, panel b, lanes 5 and 7). The same results were obtained for individual clones picked at random and for the hepta- and octa-peptide libraries (Supplementary Figure 1, panel b). An export problem was suspected because intein fragments were observed for similar cytoplasmic libraries (Supplementary Figure 1, panel c). Since the β-lactamase signal peptide is known to promote post-translational translocation of the protein across the inner membrane (*24*), we hypothesized that small intein-peptide precursors released in the cytoplasm may fold rapidly and prevent translocation, whereas the much larger intein-β-lactamase precursor is maintained in an export competent state. In order to test this hypothesis, the signal peptide was replaced by the one of DsbA (Figure 3, panel c), a signal that is known to recruit SRP and to promote cotranslational export (*25–27*). New libraries of hexa-, hepta-, and octa-peptides (pPL6-8D) were built, and as shown in Figure 3, panel a (lanes 3–6), the peptide signal change led to the observation of the DnaBI$_N$ fragment released after splicing in the periplasm. Another band cor-

TABLE 1. Diversities of the periplasmic expression libraries for cyclic hexa-, hepta-, and octa-peptides

Library	No. of transformants	Theoretical diversity	Library diversity[a]
PL6 (SX_4G)	4.2×10^6	0.16×10^6	0.16×10^6
PL7 (SX_5G)	5.8×10^6	3.2×10^6	$\sim 1.9 \times 10^6$
PL8 (SX_6G)	24×10^6	64×10^6	$\sim 15 \times 10^6$

[a]The library diversity was estimated using the GLUE-IT algorithm (*33*).

responding to the unspliced precursors was also observed at about 18 kDa. The nature of this band was confirmed by mass spectrometry. For the β-lactamase, both native and DsbA signal peptides afford the translocation and the cyclization of the enzyme in the periplasm (Figure 3, panel a, lane 6).

Combinatorial Libraries of Periplasmic Cyclic Peptides. Diversities of the libraries were evaluated by comparing the number of transformants with the theoretical number of different peptides in each library (Table 1). While the hexa-peptide library is 100% complete, the hepta- and octa-peptide libraries contain approximately 60% and 25% of the maximal diversity. Overall, the three libraries contain around 17 million different clones. The genes from 38 individual clones were sequenced and the periplasmic extracts prepared from induced cells. Thirty-five clones without a stop codon in the peptide ORF were further analyzed by SDS–PAGE. As shown in Figure 3, panel b, the presence and intensities of the bands corresponding to the $DnaBI_N$ fragment and/or the unspliced precursor were highly variable from clone to clone. As the periplasm is highly oxidant and contains an efficient catalytic machinery for disulfide formation, the rate of folding and splicing of the intein is probably competing with the rate of inactivation of its essential cysteine and explaining at least some of this clone to clone variability. The use of an intein with a catalytic serine instead of cysteine in the +1 position may solve this problem but nothing is known on the capacity of such inteins to perform cyclization in a circularly permuted state.

For eight of 38 clones, the $DnaBI_N$ fragment was not detected. The sequences of these clones (Table 2) revealed a higher frequency of tyrosines (~17.5%: 7 Tyr over 40 randomized residues) compared to the experimental codon degeneracy (4.6% of Tyr codon). Although the sampling is slightly too small for this frequency to be significative, it is correlated with a very low frequency of tyrosines in the sequences of the other 30 clones showing intein activity (1 Tyr over 138 randomized residues, Table 3). This unbalanced tyrosine representation was the only significative bias when comparing active versus inactive clones.

The periplasmic extracts of the 26 clones showing intein activity were concentrated 50 times and analyzed by MALDI-TOF-TOF mass spectrometry directly as crude samples and/or following purification on a mini C_{18} column. Results are summarized in Table 3. A more detailed table with signal-to-noise ratio and mass difference columns is provided in Supporting Information. The cyclic products $(M + H)^+$ were detected for all of the 15 peptides comprising at least one positively ionizable residue (Lys, Arg, or His) and no cysteine in their sequence. For three of them, the MS signal was the most intense of the whole spectrum indicating a relatively high concentration. Each sample served as a negative control for the other ones as well as periplasmic extracts from uninduced cells. Due to the cyclization, fragmentation spectra were hardly interpretable.

Detection, in periplasmic extracts, of individual peptides ranging from 585 to 960 Da with relatively high and similar signal-to-noise ratios (Supplementary Table 1) together with our failed attempts in detecting peptides in the extracellular medium indicate that a majority of the clones in the library biosynthesize peptides that are accumulating in this compartment and cannot diffuse outside the bacteria. Indeed, it is generally ac-

TABLE 2. Sequences of the peptides from clones with no detectable intein activity

Peptide	Sequence
P6-8	SNYWFG
P6-9	SYLIFG
P6-10	SFCVNG
P7-9	SYCYTIG
P7-10	SHSYYVG
P8-12	SHTHCALG
P8-13	STYLNKSG
P8-14	SWETNTSG

TABLE 3. MALDI TOF analysis of cyclic peptides expressed from individual library clones screened by SDS–PAGE for intein splicing

Peptide	Sequence[a]	Calculated mass	Observed mass
P6-1	S**HK**QFG	685.33	685.35
P6-2	S**K**IS**R**G	629.36	629.38
P6-3	SM**H**DGG	585.2	585.22
P6-4	SE**R**ELG	672.32	672.31
P7-1	SL**RR**GGG	684.38	684.39
P7-2	SGL**R**GVG	627.35	627.37
P7-3	SGDEP**R**G	699.3	699.31
P7-4	S**RH**WSAG	782.36	782.38
P8-1	SW**R**TQIGG	886.42	886.46
P8-2	SSSSIP**R**G	772.38	772.40
P8-3	SNF**R**WVIG	960.49	960.49
P8-4	SI**R**VQGVG	797.45	797.46
P8-5	SFG**H**ESGG	759.29	759.32
P8-6	S**R**PSLIVG	810.47	810.49
P8-7	S**H**NDTSYG	862.32	862.33
	Not detected		
P6-5	SNFSTG	594.24	
P6-6	SVTSMG	563.24	
P6-7	SLDFCG	623.24	
P7-5	SIVFLGG	674.38	
P7-6	SGGNGCG	533.17	
P7-7	SN**R**SCIG	718.32	
P7-8	S**R**C**K**F**R**G	835.42	
P8-8	SLVLNSDG	786.39	
P8-9	SVPCFA**R**G	818.39	
P8-10	SCG**R**SAVG	718.32	
P8-11	SANCGICG	706.25	

[a]Cys residues are underlined, positively ionizable residues (XH^+) are in bold type.

cepted that the outer membrane of *E. coli* is not permeable to hydrophilic solutes of more than approximately 600 Da, a limit that was evaluated using linear oligosaccharides (*28, 29*). Nonetheless, the lipid bilayer could be permeable to at least some hydrophobic peptides, and nonspecific porins of the outer membrane could also facilitate the passage of some small hydrophilic peptides, but if true, this probably concerns only a small fraction of the peptides within the libraries.

The systematic absence of signal for cyclic peptides containing no His, Lys, or Arg residues suggests that the laser energy of the spectrometer is not sufficient for their positive ionization. On the other hand, when one or more cysteine residues were encoded in the degenerated ORF, the cyclic peptides were also not detected even if basic residues were present or if the samples were reduced with DTT prior to analysis. For peptides P7-7, P7-8, P8-9, and P8-10, containing both one cysteine and at least one His, Lys, or Arg residue, we searched for Cys oxidation into sulfenic, sulfinic, and sulfonic forms, but none of those could be detected. This suggests that, if occurring, these modifications are transient. For P7-8 and P8-9 clones, however, respectively three and four fragments of the unspliced precursor were detected (Supplementary Table 2). The masses of all of these fragments indicated that the peptides contain a few additional residues essentially at the C-terminal side and a disulfide bridge resulting from the oxidation of the essential cysteine of the intein and a cysteine in the peptide. For six fragments, the whole $DnaBI_C$ has been cleaved and the observed masses correspond either to unspliced peptides cyclized by an intramolecular disulfide bridge or to backbone cyclic peptides linked to a small N-terminal fragment of $DnaBI_N$ by an intermolecular bridge. After DTT reduction and treatment with iodoacetamide, a 116 Da mass increase was observed that corresponds to a double modification on a single linear peptide therefore indicating that the peptides are unspliced. The sequences of these linear peptides were also confirmed by fragmentation analysis.

Because splicing efficiency varies from clone to clone (Figure 3, panel b), the peptides' concentrations in the periplasm should also vary and cannot be easily measured. However, the periplasmic concentration of cyclic β-lactamase was evaluated around 1 mM based on an estimation of the periplasm volume (30% of the total bacterium volume), the number of bacteria and the measure of the enzyme activity in the extract. Since the genetic constructs for peptides are very similar and the intensities of the $DnaBI_N$ fragment are in the same order of magnitude, this millimolar range is also likely for many peptides.

To summarize, we have built an expression library containing around 17 million different clones that can be divided into four groups. Approximately 50% of the clones are effectively biosynthesizing periplasmic cyclic

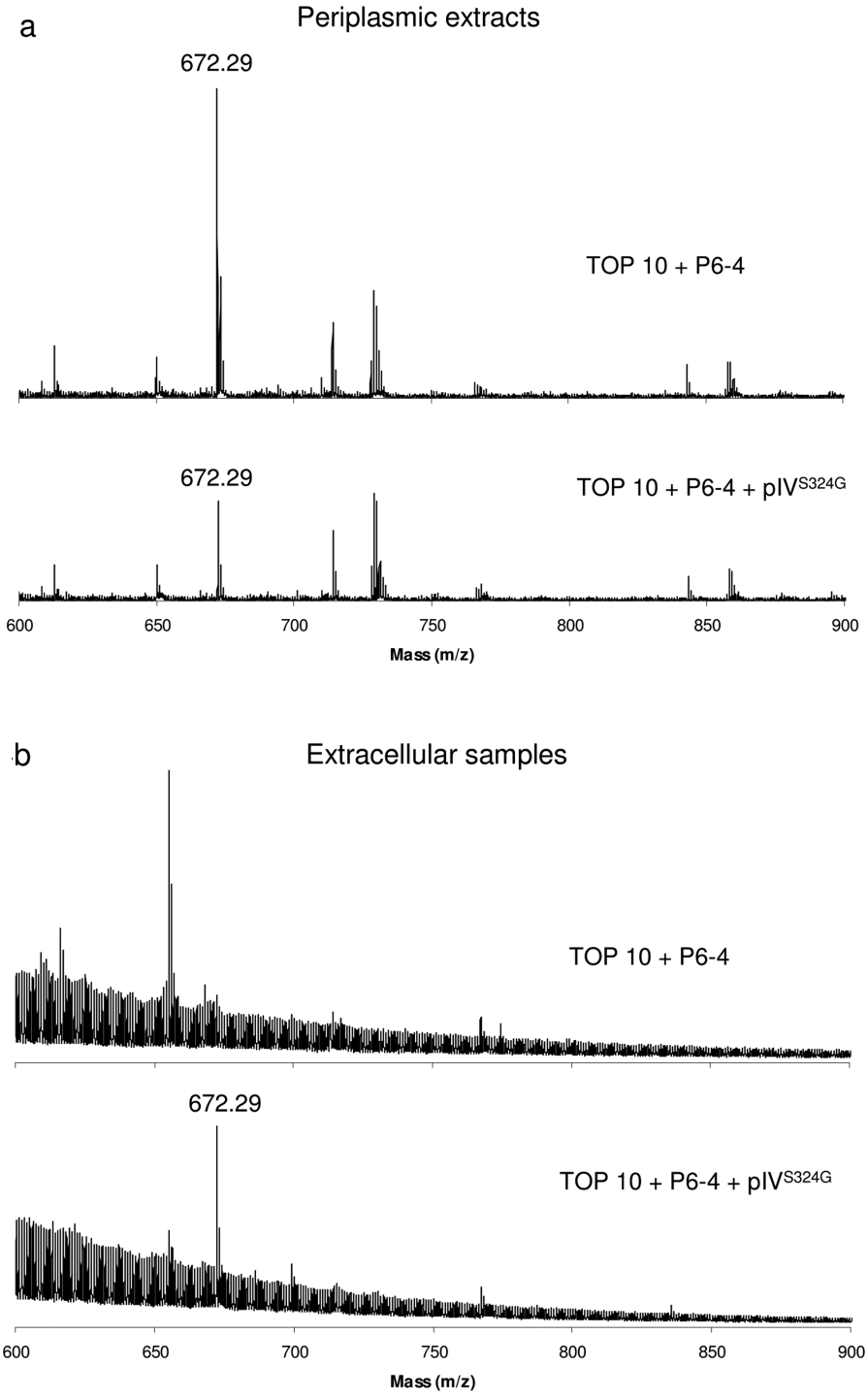

Figure 4. Secretion of a cyclic peptide in the extracellular medium. MS analysis of periplasmic (a) and extracellular (b) extracts of *E. coli* TOP10 strain expressing the P6-4 peptide (cyclic SERELG, $M + H^+ = 672.32$) with or without the coexpression of pIV^{S324G} pore protein. The periplasmic crude extracts were analyzed without any additional purification, whereas because of the high salts concentration in the culture medium, the extracellular extracts had to be desalted using a C_{18} "ZipTip" column prior to analysis.

peptides, 10% present a TAG stop codon that could eventually be translated if produced in a suppressive strain (supE), 20% are not really useful because of a translocation, splicing, or degradation problem, and about 20% are encoding peptides with one or more cysteines that are either oxidized into a disulfide bridge with the intein's essential cysteine prior to splicing or subjected to other undetected modifications. Overall, between 8 and 12 million different backbone cyclic peptides are produced in our libraries, and this diversity could be easily raised by increasing the size of the octapeptide library or creating longer peptides.

Cyclic Peptides in the Extracellular Medium. Ff bacteriophages such as F1, fd, or M13 are filamentous particles of ~7 nm diameter that use a self-encoded channel protein, pIV, to cross the outer membrane of *E. coli* without killing the bacterial cell. Marciano *et al.* (*30*) isolated a pIV mutant (pIV^{S324G}) that allows *E. coli* to grow on maltohexaose (991 g mol^{-1}) and renders the bacteria sensitive to vancomycin, a large hydrophilic antibiotic (1449 g mol^{-1}) that is normally inactive toward *E. coli* because of its incapacity to pass through the bacterial outer membrane.

We assumed that if large extracellular molecules such as vancomycin could diffuse through that pore, we should expect a similar diffusion with small cyclic peptides produced in the periplasm. To test that hypothesis, we cloned the pIV mutated gene into the low copy pPRO30 vector (*31*), which is compatible with the pBAD vector used for expressing the peptides. A tunable propionate promoter controls pIV's expression and allows setting the induction to a nontoxic level. When we coexpressed the P6-4 peptide (Table 3) with pIV^{S324G}, the peptide was easily detected in the extracellular medium (Figure 4, panel b), while its MS signal was significantly decreased in the periplasmic extract (Figure 4, panel a). In the absence of the pore, the peptide was barely detectable in the extracellular medium.

Conclusion and Perspectives. The splicing activity of the permuted Ssp DnaB mini-intein has been combined with a cotranslational export pathway to create a general method for the production of backbone cyclic proteins and peptides in the periplasm of *Escherichia coli*. With a minimal sequence requirement in the polypeptide to be cyclized, *i.e.*, a glycine and a serine as first and last residue, respectively, efficient periplasmic cyclization was demonstrated for a model protein and for a large proportion of peptides in libraries of randomized hexa-, hepta-, and octa-peptides. As these cyclic peptides are genetically encoded, selection strategies can be designed for searching molecules that bind to specific targets. The periplasmic localization opens many thrilling possibilities. First, the library can be used for searching peptides acting on colocalized targets, for instance, natural or recombinant exported proteins. Second, the selection of periplasmic peptides acting on a cytoplasmic target will automatically require the selection of their membrane permeability, which is of particular interest if looking for potential therapeutic molecules. Membrane proteins should also prove to be very attractive as targets, especially in the case of eukaryotic plasma-membrane proteins that can be functionally expressed in the inner membrane of *E. coli*. Indeed, a peptide acting from the periplasm will be equivalent to a peptide coming from outside of the eukaryotic cell that binds directly to an external site of the protein or penetrates into the membrane or into the cytosol for reaching an internal site. On the other hand, the ability to secrete cyclic peptides in the extracellular medium also opens new routes of Darwinian selection experiments such as life competition between our libraries and specific pathogens.

METHODS

Plasmid Construction. Oligonucleotides sequences with "fp" and "rp" standing for forward and reverse primer, respectively, are listed in Supporting Information. The Ssp $DnaBI_N$ and $DnaBI_C$ gene fragments were amplified from the cyanobacterium *Synechocystis* sp. strain PCC6803 (Laboratory of O. Vallon) with IN-fp/IN-rp and IC-fp/IC-rp primer pairs. The two PCR fragments were assembled by overlap extension. The final PCR product was cloned between the *NcoI* and *XbaI* sites into the pBAD vector, a derivative of pBAD/myc-HisB (Invitrogen) but carrying a tetracycline resistance gene instead of ampicillin. The C50A mutation in the $DnaBI_N$ gene was introduced by Quick-Change mutagenesis (Stratagene), resulting in the plasmid pBICA. The TEM1 β-lactamase gene was amplified with Bla-fp and Bla-rp primers encoding N-terminal and C-terminal linkers (SASGGG and GPGEFG). The product was cloned into the vector pBICA between the two BbsI sites, resulting in the plasmid pCBla. Finally, the sequences encoding either the Bla or DsbA signal peptide were introduced into the plasmid pCBla in the *NcoI* site, resulting in the final vectors pPBla and pPBlaD. For the libraries, the $DnaBI_N$ and $DnaBI_C$ gene fragments were amplified from the pBICA vector with $INL\text{-}rp/INL_{(4\text{-}6)}\text{-}fp$ and ICL-fp/ICL-rp pairs of primers. The two fragments were then restricted by BbsI, ligated together, and finally cloned between the *NcoI* and *XbaI* site into the pBAD vector containing the Bla (pPL6-8 libraries) or

DsbA (pPL6D-8D libraries) signal sequence. The pIV gene was amplified from the DNA of fd phage and the mutation S324G simultaneously added by overlap PCR. The two pairs of primers used were pIV-fp/S324G-rp and pIV-rp/S324G-fp. The primers pIV-fp and pIV-rp contain respectively a NheI and a *Bam*HI restriction sites used to clone the PCR product within the vector pPRO30 (*31*). The primer pIV-fp also codes for a Shine-Dalgarno sequence. All constructs were confirmed by sequencing.

Proteins and Peptides Expression. *E. coli* Top 10 cells (Invitrogen) harboring vectors were grown in 1 L of Luria–Bertani broth medium supplemented with tetracycline (7.5 μg mL^{-1}) at 37 °C until the culture reached an OD_{600} of 0.6. The cultures were induced with L-(+)-arabinose to a final concentration of 0.2% (w/v) and grown at 30 °C for 3 h. For the mass spectral analysis, cultures were grown in CAS medium (M9 minimal medium supplemented with casamino acids (12 g L^{-1}), tetracycline (7.5 μg mL^{-1}), L-tryptophan (2 μg mL^{-1}), $CaCl_2$ (0.1 mM), and $MgCl_2$ (1 mM) (*32*). For the experiment of cyclic peptides diffusion in the extracellular medium, the CAS medium was supplemented with ampicillin (100 μg mL^{-1}) and propionate (0.5 mM). The induction with arabinose 0.2% (w/v) was done for 3 h at 37 °C. The periplasmic extracts and the supernatants were concentrated 5 times using a SpeedVac before MS analysis.

Preparation of Extracellular, Periplasmic, and Cytoplasmic Extracts. Cultures were centrifuged at 4400 $\times$ *g* for 10 min, and supernatants were recovered as extracellular extracts. The harvested cells were resuspended in 33 mL of sucrose 20% (w/v), tris-HCl 20 mM, pH 8. After 10 min incubation time at RT, cells were centrifuged 10 min at 6000 rpm. Supernatants were discarded, and the osmotic shock was accomplished by resuspending the pellets in 50 mL of 5 mM $MgSO_4$. Samples were then incubated at 4 °C for 10 min. Supernatants obtained after centrifugation at 10 000 $\times$ *g* for 10 min were recovered as periplasmic extracts. The pellets were resuspended in 25 mL of PBS and stored overnight at −20 °C. Cells were thawed to 37 °C and centrifuged at 5400 $\times$ *g* for 10 min. Supernatants were discarded, and pellets were resuspended in 8 mL of tris-HCl 50 mM pH 8, 1 mM EDTA, and 100 mM NaCl supplemented with 100 μL of egg white lyzozyme (10 mg mL^{-1}). After 20 min of incubation time at RT followed by 45 min incubation time at 37 °C, 200 μL of DNase (1 mg mL^{-1}) was added, and lysate was incubated 15 min at RT. Supernatants obtained after centrifugation at 21 000 $\times$ g for 15 min at 4 °C were recovered as cytoplasmic extracts.

SDS–PAGE Analysis. Tris-Tricine Protein/Peptide Separation Gels were used. The cathode and anode running buffers were respectively 0.1 M Tris base/0.1 M Tricine/0.1% (w/v) SDS, and 0.2 M Tris-Cl, pH 8.9.

Cyclized β-Lactamase Purification. The cyclic β-lactamase binds to the Ni^{2+}-column thanks to two pairs of adjacent histidines present on the surface of the protein. After dialysis of the periplasmic extract, the enzyme is thus easily purified by standard affinity chromatography (HiTrap chelating, Amerscham Biosciences).

MS and MS/MS Analysis. Extracellular or periplasmic extracts were either treated as such or initially desalted using a MilliporeC18 ZipTip after acidification with 1 μL of 10% (v/v) TFA. Then 0.5 μL of each sample was added to 0.5 μL of a solution containing 10 mg mL^{-1} of α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile and 0.1% (v/v) TFA, and 0.5 μL of this mixture was put down on the target. MS and MS/MS spectra were acquired using an Applied Biosystems 4800 MALDI TOF/TOF Analyzer spectrometer using a 200 Hz solid state laser operating at 355 nm. MS spectra were obtained using a laser intensity of 3200 and 2000 laser shots by spot in a range of *m/z* between 500 and 4000. MS/MS spectra were obtained by selecting precursor ions corresponding to the precursor masses detected on the MS spectra in using a laser intensity of 3800 and 3000 laser shots by precursor. The automatically selected precursor fragmentation was performed at collision energy of 1 kV without or with collision gas air at a pressure of about 1×10^6 Torr. Data were collected with the Applied Biosystems 4000 Series Explorer software and MSMS spectra were analyzed with the Applied Biosystems GPS Explorer TM software De Novo Explorer Version 3.6.

Acknowledgment: This work was supported by the Belgian "Interuniversity Attraction Poles" program (IAP P6/19) and by the "Fonds spéciaux de recherches" of the Université Catholique de Louvain. We thank Prof. J. Keasling (University of California, Berkeley) for supplying the pPRO30 expression vector. G.D. and B.B. acknowledge FRIA fellowships from the Belgian "Fonds de la Recherche Scientifique (F.R.S.-FNRS)".

Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

REFERENCES

1. Falciani, C., Lozzi, L., Aless, Pini, R., and Bracci, L. (2005) Bioactive peptides from libraries, *Chem. Biol. 12*, 417–426.
2. Boldt, G. E., Dickerson, T. J., and Janda, K. D. (2006) Emerging chemical and biological approaches for the preparation of discovery libraries, *Drug Discovery Today 11*, 143–148.
3. Mersich, C., and Jungbauer, A. (2008) Generation of bioactive peptides by biological libraries, *J. Chromatogr., B 861*, 160–170.
4. Cane, D. E., Walsh, C. T., and Khosla, C. (1998) Harnessing the biosynthetic code: combinations, permutations, and mutations, *Science 282*, 63–68.
5. Boer, A. L. D., and Schmidt-Dannert, C. (2003) Recent efforts in engineering microbial cells to produce new chemical compounds, *Curr. Opin. Chem. Biol. 7*, 273–278.
6. Du, L., and Shen, B. (2001) Biosynthesis of hybrid peptide-polyketide natural products, *Curr. Opin. Drug Discovery Dev. 4*, 215–228.
7. Scott, C. P., Abel-Santos, E., Jones, A. D., and Benkovic, S. J. (2001) Structural requirements for the biosynthesis of backbone cyclic peptide libraries, *Chem. Biol. 8*, 801–815.
8. Scott, C. P., Abel-Santos, E., Wall, M., Wahnon, D. C., and Benkovic, S. J. (1999) Production of cyclic peptides and proteins in vivo, *Proc. Natl. Acad. Sci. U.S.A. 96*, 13638–13643.
9. Evans, T. C., Benner, J., and Xu, M. (1999) The cyclization and polymerization of bacterially expressed proteins using modified self-splicing inteins, *J. Biol. Chem. 274*, 18359–18363.
10. Tavassoli, A., and Benkovic, S. J. (2007) Split-intein mediated circular ligation used in the synthesis of cyclic peptide libraries in *E. coli*, *Nat. Protoc. 2*, 1126–1133.
11. Horswill, A. R., and Benkovic, S. J. (2005) Cyclic peptides, a chemical genetics tool for biologists, *Cell Cycle 4*, 552–555.
12. Horswill, A. R., and Benkovic, S. J. (2006) Identifying small-molecule modulators of protein-protein interactions, *Curr. Protoc. Protein Sci.* Unit 19.15.
13. Tavassoli, A., and Benkovic, S. J. (2005) Genetically selected cyclic-peptide inhibitors of AICAR transformylase homodimerization, *Angew. Chem., Int. Ed. 44*, 2760–2763.
14. Tavassoli, A., Lu, Q., Gam, J., Pan, H., Benkovic, S. J., and Cohen, S. N. (2008) Inhibition of HIV budding by a genetically selected cyclic peptide targeting the Gag-TSG101 interaction, *ACS Chem. Biol. 3*, 757–764.
15. Cheng, L., Naumann, T. A., Horswill, A. R., Hong, S., Venters, B. J., Tomsho, J. W., Benkovic, S. J., and Keiler, K. C. (2007) Discovery of antibacterial cyclic peptides that inhibit the ClpXP protease, *Protein Sci. 16*, 1535–1542.
16. Naumann, T. A., Tavassoli, A., and Benkovic, S. J. (2008) Genetic selection of cyclic peptide Dam methyltransferase inhibitors, *ChemBioChem 9*, 194–197.

17. Kinsella, T. M., Ohashi, C. T., Harder, A. G., Yam, G. C., Li, W., Peelle, B., Pali, E. S., Bennett, M. K., Molineaux, S. M., Anderson, D. A., Masuda, E. S., and Payan, D. G. (2002) Retrovirally delivered random cyclic peptide libraries yield inhibitors of interleukin-4 signaling in human B cells, *J. Biol. Chem. 277*, 37512–37518.
18. Williams, N. K., Prosselkov, P., Liepinsh, E., Line, I., Sharipo, A., Littler, D. R., Curmi, P. M. G., Otting, G., and Dixon, N. E. (2002) In vivo protein cyclization promoted by a circularly permuted *Synechocystis* sp. PCC6803 DnaB mini-intein, *J. Biol. Chem. 277*, 7790–7798.
19. Iwai, H., and Plückthun, A. (1999) Circular β-lactamase: stability enhancement by cyclizing the backbone, *FEBS Lett. 459*, 166–172.
20. Wu, H., Xu, M., and Liu, X. (1998) Protein trans-splicing and functional mini-inteins of a cyanobacterial dnaB intein, *Biochim. Biophys. Acta 1387*, 422–432.
21. Gogarten, J. P., Senejani, A. G., Zhaxybayeva, O., Olendzenski, L., and Hilario, E. (2002) Inteins: structure, function, and evolution, *Annu. Rev. Microbiol. 56*, 263–287.
22. Mathys, S., Evans, T. C., Chute, I. C., Wu, H., Chong, S., Benner, J., Liu, X. Q., and Xu, M. Q. (1999) Characterization of a self-splicing mini-intein and its conversion into autocatalytic N- and C-terminal cleavage elements: facile production of protein building blocks for protein ligation, *Gene 231*, 1–13.
23. Ludwig, C., Schwarzer, D., and Mootz, H. D. (2008) Interaction studies and alanine scanning analysis of a semi-synthetic split intein reveal thiazoline ring formation from an intermediate of the protein splicing reaction, *J. Biol. Chem. 283*, 25264–25272.
24. Koshland, D., and Botstein, D. (1982) Evidence for posttranslational translocation of beta-lactamase across the bacterial inner membrane, *Cell 30*, 893–902.
25. Powers, T., and Walter, P. (1997) Co-translational protein targeting catalyzed by the Escherichia coli signal recognition particle and its receptor, *EMBO J. 16*, 4880–4886.
26. Schierle, C. F., Berkmen, M., Huber, D., Kumamoto, C., Boyd, D., and Beckwith, J. (2003) The DsbA signal sequence directs efficient, cotranslational export of passenger proteins to the *Escherichia coli* periplasm via the signal recognition particle pathway, *J. Bacteriol. 185*, 5706–5713.
27. Luirink, J., and Sinning, I. (2004) SRP-mediated protein targeting: structure and function revisited, *Biochim. Biophys. Acta 1694*, 17–35.
28. Nakae, T., and Nikaido, H. (1975) Outer membrane as a diffusion barrier in Salmonella typhimurium. Penetration of oligo- and polysaccharides into isolated outer membrane vesicles and cells with degraded peptidoglycan layer, *J. Biol. Chem. 250*, 7359–7365.
29. Decad, G. M., and Nikaido, H. (1976) Outer membrane of Gram-negative bacteria. XII. Molecular-sieving function of cell wall, *J. Bacteriol. 128*, 325–336.
30. Marciano, D. K., Russel, M., and Simon, S. M. (1999) An aqueous channel for filamentous phage export, *Science 284*, 1516–1519.
31. Lee, S. K., and Keasling, J. D. (2005) A propionate-inducible expression system for enteric bacteria, *Appl. Environ. Microbiol. 71*, 6856–6862.
32. Soumillion, P., and Fastrez, J. (1992) A large decrease in heat-shock-induced proteolysis after tryptophan starvation leads to increased expression of phage lambda lysozyme cloned in *Escherichia coli*, *Biochem. J. 286*, (Pt 1), 187–191.
33. Patrick, W. M., Firth, A. E., and Blackburn, J. M. (2003) User-friendly algorithms for estimating completeness and diversity in randomized protein-encoding libraries, *Protein Eng. 16*, 451–457.