Unprecedented Rates and Efficiencies Revealed for New Natural Split Inteins from Metagenomic Sources*

Received for publication, April 17, 2012, and in revised form, June 28, 2012 Published, JBC Papers in Press, June 28, 2012, DOI 10.1074/jbc.M112.372680

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Background: Hypothetical natural split inteins from environmental metagenomic sources were evaluated experimentally for the first time.

Results: Four inteins showed the highest known reaction rates and efficiencies for protein trans-splicing and could be used efficiently for C-cleavage.

Conclusion: These inteins pushed the catalytic limit of protein trans-splicing, although this was unpredictable from their primary sequence.

Significance: These inteins hold great potential as versatile protein engineering tools.

Inteins excise themselves out of precursor proteins by the protein splicing reaction and have emerged as valuable protein engineering tools in numerous and diverse biotechnological applications. Split inteins have recently attracted particular interest because of the opportunities associated with generating a protein from two separate polypeptides and with trans-cleavage applications made possible by split intein mutants. However, natural split inteins are rare and differ greatly in their usefulness with regard to the achievable rates and yields. Here we report the first functional characterization of new split inteins previously identified by bioinformatics from metagenomic sources. The N- and C-terminal fragments of the four inteins gp41-1, gp41-8, NrdJ-1, and IMPDH-1 were prepared as fusion constructs with model proteins. Upon incubation of complementary pairs, we observed trans-splicing reactions with unprecedented rates and yields for all four inteins. Furthermore, no side reactions were detectable, and the precursor constructs were consumed virtually quantitatively. The rate for the gp41-1 intein, the most active intein on all accounts, was $k = 1.8 \times 10^{-1} \text{ s}^{-1}$, which is ~10-fold faster than the rate reported for the Npu DnaE intein and gives rise to completed reactions within 20–30 s. No cross-reactivity in exogenous combinations was observed. Using C1A mutants, all inteins were efficient in the C-terminal cleavage reaction, albeit at lower rates. C-terminal cleavage could be performed under a wide range of reaction conditions and also in the absence of native extein residues flanking the intein. Thus, these inteins hold great potential for splicing and cleavage applications.

Inteins, a combination of the words “internal protein” (1), were first discovered in 1990 when it was observed that the translation of the VMA1 gene of *Saccharomyces cerevisiae* did not result in the predicted 120-kDa protein, but a much shorter polypeptide chain of 69 kDa (2). To obtain the mature 69-kDa protein, an internal part of the larger precursor had to be excised and the flanking regions, called N- and C-extein, rejoined in an autocatalytic post-translational process termed protein splicing. Furthermore, the excised intein fragment contained a nonessential part exhibiting homing endonuclease activity that could be deleted without affecting the overall ability of the mini-intein to cleave and splice (3). More recently, inteins without the endonuclease domain have also been found (4). Since that time, almost 500 other inteins have been identified in the three kingdoms of organisms (archaea, bacteria, and eukaryotes), and their sequences have been collected in a dedicated database (5). Overall, four short conserved regions on the amino terminus and two conserved domains on the carboxyl terminus could be identified that can be used to search for novel inteins (1, 6). Besides the inteins that are encoded in a single gene, a few split inteins were also discovered and characterized biochemically that are transcribed and translated from two separate genes and lack the endonuclease domain. These include a number of alleles inserted in the cyanobacterial DnaE gene encoding the α subunit of DNA polymerase (7), such as Npu DnaE (8), Ssp DnaE (9), and *Anabaena* sp. DNA inteins (10) as well as the archaeon *Neg* polymerase intein (11). As shown in Fig. 1A, the translation of both fragments reconstitutes the catalytic activity of protein trans-splicing to form the mature protein (12). Split inteins can also be generated artificially by dividing inteins into two or three fragments (13). In the case of the Ssp DnaB intein, the N-terminal intein fragment (IntN)3 could be as short as 11 amino acids (14), whereas 6 amino acids were sufficient for the IntC fragment of the Ssp GyrB intein (15).

Because inteins are in principle tolerant with regard to heterologous sequences at their flanking extein regions, they can be inserted into other proteins. This opens the door for many

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*This work was supported by ERA BIOTECH®.
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3 The abbreviations used are: IntN, intein-N; IntC, intein-C; Saa, native extein-N; Saa2, native extein-C; ST, Steep-tag II; gpD, bacteriorhodopsin λ head protein D; Trx, thioredoxin; H5, His tag; SP, splice product; IMPDH, inosine-5’-monophosphate dehydrogenase; Bis-Tris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propane-1,3-diol.
applications, for instance, to generate cyclic peptides (16), to generate protein thioesters (17), to label proteins (18, 19), and probably most frequently, to cleave off affinity tags for protein purification (20). Particularly to optimize cleavage efficiency, inteins have been mutagenized to become inducible by pH (21), reducing agents (22), or temperature (23) and to separate cleavage activity from splicing. Interestingly, inteins can be engineered to cut either at the amino terminus (22) or at the carboxyl terminus (24).

Many of the artificial split inteins require refolding or show low solubility in fusion constructs, which limits their application. However, split inteins are especially attractive for biotechnological applications because they have no premature activity when expressed separately and they are much smaller than normal inteins and thus potentially have less negative impact on the expression level of the fused target protein (25). The ideal intein for some biotechnological applications would therefore be a split intein that is highly active without the need of refolding. Currently, the Npu DnaE intein appears to be the most advantageous split intein as it displays superior splicing kinetics and high efficiency (8, 26).

After the first isolation of naturally occurring split inteins, efforts were undertaken to find more native split inteins. Most recently, a search in Global Ocean Sampling (GOS) environmental metagenomic sequence data was performed to find fractured genes that contain novel split inteins (27). These fractured genes code for potentially essential cellular proteins, and in the case of four insertion sites (gp41 DNA helicase, inosine-5’-monophosphate dehydrogenase (IMPDH), ribonucleotide reductase catalytic subunit NrdJ), and DnaE polymerase II subunit α), the complete loci including the split inteins could be assembled. However, it remained unclear whether the inteins were active in protein trans-splicing.

The purpose of this study was to functionally characterize four representatives of these novel hypothetical split inteins (gp41-1, gp41-8, NrdJ-1, and IMPDH-1) in the protein trans-splicing reaction and to perform mutagenesis to evaluate their efficiency and applicability for trans-cleavage. We found that these inteins exhibit an unprecedented activity in terms of reaction speed and efficiency.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Gene sequences and specific details of novel split inteins gp41-1, gp41-8, NrdJ-1, and IMPDH-1 are already described elsewhere (27). Gene synthesis of split intein fragments was performed by Biomatik (Cambridge, Canada), including flanking restriction sites for easy subcloning into the previously described pAU08 and pVS01 plasmids (26). Construction of the expression plasmids encoding the IntC fragments of gp41-1, gp41-8, NrdJ-1, and IMPDH-1 together with their 5 native extein residues (5aaC) were obtained by KpnI and NdeI restriction digestion and by their subcloning into pAU08 to replace the Npu DnaEN; an ST-gpD sequence is encoded upstream of the IntN fragments. A C1A single-point mutation to allow C-terminal cleavage was performed in constructs containing the IntN fragments. An ST-gpD sequence is encoded upstream of the IntC fragments. The four generated expression plasmids were named as follows: gp41-1C, gp41-8C, NrdJ-1C, and IMPDH-1C. Plasmids encoding the IntC split intein fragments of gp41-1, gp41-8, NrdJ-1, and IMPDH-1 together with their 5

native extein residues (5aaC) were obtained by KpnI and NdeI (Fermentas, St. Leon-Rot, Germany) restriction digestion and by their subcloning into pVS01 to replace the Npu DnaEC. A Trx-His tag sequence is downstream of the IntC fragments. The four generated expression plasmids were named as follows: gp41-1C, gp41-8C, NrdJ-1C, and IMPDH-1C.

Single-point mutation to allow C-terminal cleavage was performed in constructs containing the IntN fragments. A C1A mutation for each split intein was performed by PCR using specific primers to give gp41-1C(C1A), gp41-8C(C1A), NrdJ-1C(C1A), and IMPDH-1C(C1A). Synthetic oligonucleotides were obtained from Thermo Scientific (Ulm, Germany).
To permit a clear C-terminal cleavage, 5aaC residues placed between the IntC and the thioredoxin (Trx) encoding gene sequence were removed by PCR using specific primers to give gp41-1Cext), gp41-8Cext), NrdJ-1Cext), and IMPDH-1Cext). All plasmids were verified by DNA sequencing (Macrogen). Table 1 gives an overview of all constructs used in this study and their numbering.

**Expression of the Recombinant Intein Fusion Proteins**—All generated plasmids were used for transformation of *Escherichia coli* BL21 (DE3) cells (Stratagene). Cells were grown at 37 °C and 250 rpm in shake flasks containing 600 ml of LB medium supplemented with the corresponding plasmid maintenance antibiotics (50 μg/ml kanamycin or 100 μg/ml ampicillin), until A600 nm reached ~0.6. Induction of gene expression was then carried out in the presence of 0.4 mM isopropyl-β-D-thiogalactopyranoside for the IntN constructs or by the addition of 0.02% L-arabinose for the IntC constructs; in both cases, the temperature was lowered to 28 °C. After a 5-h induction, cells were harvested by centrifugation for 15 min at 5000 rpm, and the cell pellet was stored at −80 °C.

**Split Intein Fusion Protein Purification**—Protein purification was always performed under native conditions. For the IntN constructs, the cell pellet was resuspended in buffer containing 100 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA. For the IntC constructs, cells were resuspended in buffer containing 50 mM Tris/HCl (pH 8.0), 300 mM NaCl, and 20 mM imidazole. Cells were sonicated on ice for 20 min with a 30% pulsed activity (Branson 250 Sonifier) and centrifuged for 30 min at 30000 × g at 4 °C. The soluble fraction of IntC constructs, cells were resuspended in buffer containing 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, and 10% (v/v) glycerol) in the presence of 2 mM DTT, and stored in aliquots at −80 °C. Protein concentration was determined using the calculated absorbance at 280 nm.

**Table 1**

| Protein          | Abbreviation | Protein sequence* | Molecular mass |
|------------------|--------------|-------------------|----------------|
| gp41-1N          | MASWSHPQFKEAS-gpD-GS-[TRSGY]-gp41-1N-GGHHHHHHHH | 24.2 kDa |
| gp41-8N          | MASWSHPQFKEAS-gpD-GS-[QLNR]-gp41-8N-GGHHHHHHHH | 24.2 kDa |
| NrdJ-1N          | MASWSHPQFKEAS-gpD-GS-[GTNPC]-NrdJ-1N-GGHHHHHHHH | 26.2 kDa |
| IMPDH-1N         | MASWSHPQFKEAS-gpD-GS-[GQGQ]-IMPDH-1N-GGHHHHHHHH | 25.8 kDa |
| Npu DnaE2        | gp41-1T-GS-SSSPDT-GT-Trx-EFRRSHHHHH | 18.8 kDa |
| gp41-8T          | gp41-8T-SAVE-GT-Trx-EFRRSHHHHHHHH | 19.1 kDa |
| NrdJ-1T          | NrdJ-1T-SEIV-GT-Trx-EFRRSHHHHHH | 18.6 kDa |
| IMPDH-1T         | IMPDH-1T-SWCT-GT-Trx-EFRRSHHHHH | 18.6 kDa |
| Npu DnaE1        | Npu DnaE1-SCT-GT-Trx-EFRRSHHHHH | 17.9 kDa |
| gp41-1N(C1A)     | MASWSHPQFKEAS-gpD-GS-[TRSGY]-gp41-1N(C1A)-GGHHHHHHHH | 24.2 kDa |
| gp41-8N(C1A)     | MASWSHPQFKEAS-gpD-GS-[QLNR]-gp41-8N(C1A)-GGHHHHHHHH | 24.2 kDa |
| NrdJ-1N(C1A)     | MASWSHPQFKEAS-gpD-GS-[GTNPC]-NrdJ-1N(C1A)-GGHHHHHHHH | 26.2 kDa |
| IMPDH-1N(C1A)    | IMPDH-1N(C1A)-GGHHHHHH | 25.8 kDa |
| Npu DnaE3        | gp41-1T-GT-Trx-EFRRSHHHHHH | 18.3 kDa |
| gp41-8T(C1A)     | gp41-8T-GT-Trx-EFRRSHHHHHH | 18.6 kDa |
| NrdJ-1T(C1A)     | NrdJ-1T-GT-Trx-EFRRSHHHHHH | 18.2 kDa |
| IMPDH-1T(C1A)    | IMPDH-1T-GT-Trx-EFRRSHHHHHH | 18.1 kDa |

*Native extein sequences are in brackets, StreptagII- and His-tag are underlined.

**Table 2**

| Protein Constructs Used in This Study | Molecular Mass |
|--------------------------------------|----------------|
| gp41-1N                              | 24.2 kDa       |
| gp41-8N                              | 24.2 kDa       |
| NrdJ-1N                              | 26.2 kDa       |
| IMPDH-1N                             | 25.8 kDa       |

**RESULTS**

**Generation and Purification of Split Intein Fusion Constructs**—The four split inteins gp41-1, gp41-8, NrdJ-1, and IMPDH-1 were prepared as IntN and IntC fusion partners for the *in vitro* analysis. As shown in Fig. 1B, all the fusion proteins contained...
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Time-dependent in Vitro trans-Splicing Analysis—Protein splicing is a self-processing pathway mediated by the intein domain. In the most common class I inteins (28), four successive nucleophile displacement reactions that directly involve the side chain functional groups of three of the four splice junction residues lead to intein excision with concomitant ligation of the N- and C-terminal exteins. In protein trans-splicing, this pathway is preceeded by intein fragment association, as depicted in Fig. 1A. Purified IntN fusions (proteins 1–5 in Table 1) and IntC fusions (proteins 6–10 in Table 1) were mixed in pairwise combinations in a 1:1 stoichiometry. For each intein, the spontaneous trans-splicing reaction was monitored at different temperatures for the formation of the splice product (SP) ST-gpDTrx-H4 and the consumption of the IntN and IntC precursors in SDS-PAGE gels. Also, the formation of the excised IntN and IntC fragments could be observed (compare Fig. 1B). The identity of the expected SP sequence was confirmed in each case by immunoblotting with anti-Strep-tag II and anti-Trx (data not shown). Moreover, the SP and the trypsin fragment covering the new peptide bond linking the exteins were identified by LC-MS/MS (data not shown).

To determine the kinetics of the reactions, aliquots were removed at the specified time points and immediately quenched by mixing with SDS sample buffer and boiling. Surprisingly, the four new split inteins showed extremely fast trans-splicing reactions. In particular, the gp41-1 intein exhibited outstanding splice kinetics and yields with optimal performance at 45 °C. As shown in Fig. 2A, already within the first 3 s, the two precursor proteins disappeared almost completely (bands 1 and 6), whereas the SP and the IntN and IntC halves were formed. The other inteins gp41-8, NrdJ-1, and IMPDH-1 also spliced exceptionally fast and efficiently. After 1 min of incubation at their optimal temperature (37 °C), the respective precursors (Fig. 2B, bands 2 and 7, 3 and 8, and 4 and 9) were nearly depleted, and the SP as well as the IntN and IntC halves were detected as the predominant bands in the SDS-PAGE gels. For comparison, the previously described Npu DnaE intein was examined at its optimal temperature of 37 °C (26). Fig. 2C shows that SP formation occurred at a lower rate than seen for the new split inteins. For instance, after 1 min, the precursors are converted to the products to only about 50%.

For a more quantitative analysis, rates (k) and yields (in percentage) from the time course experiments were determined under different temperatures (Fig. 3). Rates were fitted to an exponential first-order equation with the assumption that the sequence of bacteriophage λ head protein D (gpD) and thioredoxin (Trx) as N- and C-exteins, respectively. Strep-tag II (ST) and/or His tag (H4) were added for purification purposes. Five native extein amino acids (5aa) were kept upstream and downstream of the IntN and IntC fragments. Additionally, the non-native amino acid sequence GS upstream of the native ExteinN residues and GT downstream of the native ExteinC residues were added due to cloning reasons (Table 1). All proteins were expressed in soluble form in E. coli and purified under native conditions. The Npu DnaE intein, considered to have the highest splicing rate yet described, was used as a control in this study with previously described constructs (26) that exhibit equivalent fusion protein design (Fig. 1B and Table 1).

FIGURE 2. Analyses of protein trans-splicing reactions. Shown are Coomassie Brilliant Blue-stained SDS-gels (4–12% acrylamide). In all cases, the precursor proteins (indicated by numbers) were converted into the SP and the excised intein fragments IntN and IntC. Samples at t = 0 min are precursor proteins that were boiled before mixing. A, time-dependent analysis of the gp41-1 intein reaction performed at 45 °C. M denotes molecular marker. B, analyses of the reactions involving the gp41-8, NrdJ-1m and IMPDH-1 inteins performed at 37 °C. The band marked with an asterisk is a protein contamination. C, time-dependent analysis of the Npu DnaE intein reaction performed at 37 °C. The band marked with an asterisk denotes a protein impurity, whereas the band marked with a number sign corresponds to the N-terminal cleavage product (ST-gpD) resulting from cleavage of the linear thioester by β-mercaptoethanol in the SDS-sample buffer used to quench the reaction (as observed previously in Ref. 26).
The optimal temperature for the other three inteins was 37 °C (Fig. 3, A and C, right panel), at which the gp41-8 intein displayed a \( k = (4.5 \pm 0.6) \times 10^{-2} \) s\(^{-1}\) with a calculated \( t_{1/2} = 15 \) s (85–95% yield). The NrdJ-1 intein had a \( k = (9.8 \pm 2.3) \times 10^{-2} \) s\(^{-1}\) with \( t_{1/2} = 7 \) s (85–95% yield), and the IMPDH-1 intein had a \( k = (8.7 \pm 3.2) \times 10^{-2} \) s\(^{-1}\) with \( t_{1/2} = 8 \) s (90–95% yield) (Table 2). These inteins are thus ~3-, 7- and 6-fold faster than the Npu DnaE. In Fig. 3C, the left panel shows the gp41-1 intein at its optimal temperature for comparison. Considering their highest rates, these inteins can be arranged in a descending order as follows: gp41-1 > NrdJ-1 > IMPDH-1 > gp41-8 > Npu DnaE.

**Orthogonality of the Split Inteins**—To determine the interaction specificity, fusion constructs with the N- and C-terminal halves of the four new split inteins were mixed in all possible 16 combinations, resulting in four endogenous (wild type) and 12
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To assess the potential of the new split inteins as protein splicing tools, we mutated the Cys-1 to Ala at the N-terminal splice junction of the four inteins, gp41-1, gp41-8, NrdJ-1, and IMPDH-1 (proteins 11–14; Fig. 1B and Table 1). The purified mutant fusion constructs were incubated with the previously used C-terminal constructs 6–9, containing Trx as the product of interest and the 5 native C-extein residues flanking the intein (Fig. 1B and Table 1). C-cleavage activity at different temperatures (0, 25, and 37 °C) was determined by quantifying the protein bands by SDS-PAGE as described before.

In all the cases, C-cleavage activity with good (40–50%) to excellent (about 85–95%) yield was detected (Table 4), albeit at lower rates than observed for the trans-splicing reactions (Table 2). The corresponding SDS-PAGE gels are shown in Fig. 4, A–D, left panels. In each case, the bands of the precursor protein (bands 6, 7, 8, and 9 respectively) were cleaved into two products: the 5aaC-Trx protein and the respective IntC fragment. At 37 °C, the gp41-1 intein exhibited the highest C-cleavage rate among the new inteins k = (2.2 ± 0.7) × 10⁻³ s⁻¹, corresponding to a calculated t½ = 5 min with 85–95% yield followed by the IMPDH-1 intein k = (7.7 ± 1.6) × 10⁻² s⁻¹, t½ = 15 min (85–95% yield), the NrdJ-1 intein (2.4 ± 0.1) × 10⁻¹ s⁻¹, t½ = 48 min (85–95% yield), and the gp41-8 intein (7.7 ± 1.5) × 10⁻⁵ s⁻¹, t½ = 150 min (40–50% yield). The precise C-cleavage was confirmed by Edman sequencing of the liberated 5aaC-Trx (data not shown).

Table 4 also shows the effect of temperature on the C-cleavage reaction. Lower rates were associated with lower temperatures, but the yields were only slightly compromised. For example, at 0 °C, the gp41-1, NrdJ-1, and IMPDH-1 inteins showed yields as high as at 37 °C (>80%), meaning that cleavage efficiencies are robust over a wide temperature range. On the other hand, the gp41-8 intein generally showed lower rates with lower yields (<50%). In addition, analysis of the pH influence indicated that pH values above 7 resulted in a decrease of C-cleavage efficiency (data not shown). The best C-cleavage yields and constant rates under the evaluated conditions were obtained at 37 °C and pH 7 (see also Fig. 4E).

In summary, the four new inteins could be used for C-terminal cleavage reactions by introducing a C1A mutation, although the rates and in some cases the yields were markedly reduced when compared with the trans-splicing reaction. This finding is in contrast to the naturally split inteins Npu DnaE and Ssp DnaE whose C-terminal cleavage reaction is completely blocked in the context of a C1A mutation (12, 26).

C-cleavage without Any Extra Amino Acids and Effect of Native Exttein Sequence—For practical applications, it is often highly desirable to have a clean cleavage without any additional amino acid attached to the product of interest. We generated four clones without the 5 residues corresponding to the native exogenous combinations. Protein splicing was only detected in the endogenous combinations, showing that the inteins are orthogonal (data not shown).

Effect of Urea and pH on gp41-1 trans-splicing—We tested the effect of a chaotropic salt and different pH conditions (pH 6–10) on trans-splicing of the gp41-1 intein (Table 3). Prior to mixing of the complementary intein constructs, the samples were equilibrated in the corresponding buffer conditions as indicated. gp41-1 intein activity remained very high in the presence of 4 M urea (k = 1.0 × 10⁻³ s⁻¹; yield = 80–85%), but decayed 50-fold with 6 M urea. There was no activity detected at 8 M urea. The gp41-1 intein was robust toward the pH variation, and the rates and yields remained similar from pH 6–10 (Table 3).

C-cleavage Induction with Point Mutation of Conserved Residues—Cleavage mediated by inteins is attractive for some biotechnological applications, e.g. bioprocess operations to release the protein of interest from a fusion partner. For this application, the intein must be altered to avoid trans-splicing and to effect a controllable cleavage. The mutation of the first Cys residue of an intein typically abolishes step 1 (the N–S acyl shift), step 2 (formation of the branched intermediate by attack of the first residue in the C-extein), and step 4 (rearrangement to a peptide bond by a spontaneous O–N acyl shift) of the splicing reaction (Fig. 1A). In such mutated inteins, step 3 (Asn cyclization) may still occur, leading to C-terminal cleavage of the C-extein from the N-extein/intein portion.

To assess the potential of the new split inteins as protein cleavage tools, we mutated the Cys-1 to Ala at the N-terminal splice junction of the four inteins, gp41-1, gp41-8, NrdJ-1, and IMPDH-1 (proteins 11–14; Fig. 1B and Table 1). The purified mutant fusion constructs were incubated with the previously

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**Table 2**

Apparent first order rate constants and yields of trans-splicing reactions - temperature dependence

| Temperature (°C) | gp41-1(1±6) | gp41-8(2±7) | NrdJ(1±3+8) | IMPDH(1±4+9) | Npu DnaE(5±10) |
|-----------------|------------|------------|-------------|-------------|----------------|
|                 | k          | Y%         | k           | Y%          | k              |
| 0               | (5.1 ± 0.8) × 10⁻³ | 75–85 | (7.7 ± 1.5) × 10⁻² | 85–95 | (3.6 ± 1.4) × 10⁻² | 90–95 |
| 12              | (1.8 ± 0.7) × 10⁻² | 80–90 | (9.8 ± 2.3) × 10⁻² | 85–95 | (8.7 ± 3.2) × 10⁻² | 90–95 |
| 25              | (5.7 ± 0.6) × 10⁻² | 80–90 | (5.3 ± 2.8) × 10⁻² | 75–85 | (7.9 ± 1.9) × 10⁻² | 90–95 |
| 37              | (1.4 ± 0.2) × 10⁻¹ | 90–95 | (1.2 ± 0.1) × 10⁻² | 35–45 | (3.6 ± ND) × 10⁻² | 85–95 |
| 45              | (1.8 ± 0.5) × 10⁻¹ | 85–95 | (9.0 ± ND) × 10⁻² | 30–35 | (9.7 ± ND) × 10⁻³ | 80–90 |
| 50              | (1.2 ± 0.2) × 10⁻¹ | 85–95 | (8.8 ± ND) × 10⁻⁻ | 20–30 | ND |
| 55              | (8.3 ± 1.3) × 10⁻² | 65–75 | ND |
| 60              | (1.0 ± 0.2) × 10⁻² | 40–50 | ND |

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**Table 3**

Effect of pH and urea on gp41-1 trans-splicing at 25 °C

| pH | Urea | k | t½ | Y% |
|----|------|---|----|----|
| 6  | 0    | (4.0 ± 0.2) × 10⁻² | 17 s | 80–90 |
| 8  | 0    | (5.4 ± 0.2) × 10⁻² | 13 s | 80–90 |
| 9  | 0    | (4.1 ± 0.8) × 10⁻² | 17 s | 80–90 |
| 10 | 0    | (4.5 ± 1.2) × 10⁻² | 16 s | 80–90 |
| 7  | 4    | (1.0 ± ND) × 10⁻² | 11 min | 80–85* |
| 6  | 7    | (2.0 ± ND) × 10⁻⁵ | 577 min | 30–35* |

* 24-h time course.
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C-extein sequence (proteins 15–18) and tested for C-cleavage activity when the purified proteins were mixed with the complementary IntN C1A fusions (proteins 11–14). In Fig. 4, the right panels show these cleavage reactions for each intein. Fig. 4A, right panel, the complete time course of the C-cleavage mediated by the gp41-1 intein is shown. The IntN precursor (band 15) is almost completely cleaved, liberating the Trx (without any extra amino acid) and IntN fragment. For all the inteins, the predicted N terminus of the Trx cleavage product was confirmed by Edman sequencing (data not shown).

The optimal temperature for each intein was determined. As can be seen in Table 5, in general, the four split inteins showed high thermostability over a wide range of temperatures (0–55 °C), with the highest C-cleavage activities between 37 and 50 °C.

The deletion of the 5 native C-extein residues affected the rates and yields of the four split inteins at varying degrees (Tables 4 and 5, Fig. 4E). At 37 °C, the gp41-1 intein had a 10-fold rate reduction but maintained its excellent yields (85–95%). The gp41-8 intein was again associated with lower yields (20–30%), and it had a 1.5-fold rate reduction, whereas the NrdJ-1 intein had a 5-fold rate reduction and a drop to 60–70% yield. The IMPDH-1 intein was severely affected, having a 38.5-fold rate reduction and a drop to 65–75% yield. Similar to the trans-splicing results (Table 2), the gp41-1 and IMPDH-1 inteins were active even at 60 °C, whereas the gp41-8 and NrdJ-1 inteins were inactive at temperatures higher than 50 °C.

In conclusion, under all evaluated conditions, the gp41-1 intein possessed the highest rates and yields among the four inteins. It can be considered as the most robust split intein for C-cleavage without adding extra native amino acids.

**DISCUSSION**

Split inteins hold a tremendous potential for a wide range of protein engineering approaches (17, 19, 30). Recently, novel biotechnological applications for split inteins were described. They represent a combination of affinity binding and cleavage for protein purification (31), the generation of antibody toxin conjugates (32), or widespread use as tools in drug discovery (33). In general, intein-tagged fusion protein systems enhance the efficiency of protein affinity purification processes by way of requiring only a single chromatographic step (34). Thus, there is a great need for novel split inteins with beneficial biochemical properties.

We characterized for the first time the functionality, in terms of protein splicing and self-cleavage activities, of four novel hypothetical split inteins, gp41-1, gp41-8, NrdJ-1, and IMPDH-1, previously predicted from metagenomic sequences (27). All pairs of split intein protein fragments were assessed as model fusion proteins under identical extein context to allow their comparison with the previously reported extremely efficient and fast-splicing split intein Npu DnaE (26). Splicing rates of the gp41-1, gp41-8, NrdJ-1, and IMPDH-1 inteins were 10-, 3-, 7-, and 6-fold higher, respectively, than those reported for the Npu DnaE at 37 °C, and all of them showed excellent (90–95%) splicing yields (Table 2) with no apparent side-product formation. It is also noteworthy that both precursor halves were nearly quantitatively consumed in the reactions. Our results for the Npu DnaE trans-splicing rates were similar to previous studies (26), confirming that the observed rate differences cannot be accounted for by different laboratory conditions of any kind.

To further characterize the naturally split inteins, we tested their splicing activity over a wide range of temperatures (0–60 °C). Interestingly, we found that the optimal temperature for the gp41-1 split intein was around 45 °C, whereas for gp41-8, NrdJ-1, and IMPDH-1 inteins, it was 37 °C (Fig. 3). The functional thermostability of these split inteins might be explained by the environmental conditions of the sampling site. The genes corresponding to all four novel split inteins were identified from an environmental microbial source GOS metagenomic data sampled in Lake Gatun (Panama), near the tropical Caribbean Sea, with water temperatures of 28.5 °C (35). The gp41-1 intein was found to be most robust and gave excellent yields at a wide range of conditions, including different alkali pHs, high temperatures such as 60 °C, and the presence of chaotropic agents.

We observed an absence of promiscuity in different exogenous intein fragment combinations, i.e. only the complementary pairs of inteins fragments were active. Such orthogonality would hinder the functional complementation of different split inteins inside the same host cell and therefore would not exclude the possibility that some or all of these inteins are from the same microbial origin. However, given their isolation from large-scale metagenomic sequencing efforts, it appears more likely that the four inteins are derived from different organisms.

For some biotechnological applications, this orthogonality could be highly valuable as it allows their simultaneous use, for example, for three-piece ligations (29, 36). In contrast, most exogenous combinations of cyanobacterial DnaE intein alleles exhibit efficient cross-splicing activity (7, 8) and are therefore less suited for such purposes.

Another example of a potential application for the new split inteins is protein labeling using the Cys tag approach, in which
FIGURE 4. C-cleavage with and without native extein amino acids. For these reactions at 37 °C, a C1A mutation was introduced into each IntN fusion protein. 5 native C-extein amino acids (5aaC) were either included (left panels) or deleted (right panels). A–D, shown are Coomassie Brilliant Blue-stained SDS-gels. The C-terminal precursor proteins (indicated by numbers) were either completely or partially converted into the ExteinC (indicated as Trx in all cases) and the InteinC fragment. The N-terminal precursor protein remained unchanged during the reaction. Proteins bands marked with an asterisk denote protein impurities. M denotes molecular marker. E, C-cleavage constant rates of the four inteins determined at 25 and 37 °C. Error bars indicate S.D.
Unprecedented Fast Split Inteins

**TABLE 5**

Apparent first order rate constants and yields of C-terminal cleavage without any native ExtC amino acids

| Temperature | gp41-1 (11 + 15) | gp41-8 (12 + 16) | NrdJ-1 (13 + 17) | IMPDH-1 (14 + 18) |
|-------------|-----------------|-----------------|-----------------|-----------------|
| °C | $k$ | $Y$ | $k$ | $Y$ | $k$ | $Y$ | $k$ | $Y$ |
| 25 | $(6.7 \pm 2.5) \times 10^{-5}$ | 85–95 | $(1.0 \pm ND) \times 10^{-5}$ | 10–15 | $(1.4 \pm 0.8) \times 10^{-5}$ | 30–40 | $(5.7 \pm 0.2) \times 10^{-6}$ | 15–25 |
| 37 | $(2.3 \pm 0.1) \times 10^{-4}$ | 90–95 | $(5.2 \pm 2.5) \times 10^{-5}$ | 20–30 | $(4.9 \pm 2.0) \times 10^{-5}$ | 60–70 | $(2.0 \pm 0.0) \times 10^{-5}$ | 65–75 |
| 45 | $(4.4 \pm 1.2) \times 10^{-4}$ | 90–95 | $(6.7 \pm 1.5) \times 10^{-5}$ | 20–30 | $(5.1 \pm 0.2) \times 10^{-5}$ | 70–80 | $(2.8 \pm 0.1) \times 10^{-5}$ | 70–80 |
| 50 | $(4.3 \pm 0.5) \times 10^{-4}$ | 85–95 | $(6.1 \pm 0.1) \times 10^{-5}$ | 20–30 | $(6.0 \pm ND) \times 10^{-6}$ | 10–15 | $(3.5 \pm 0.7) \times 10^{-5}$ | 80–90 |
| 55 | $(1.9 \pm 1.0) \times 10^{-4}$ | 65–85 | NA | NA | NA | $(8.0 \pm ND) \times 10^{-6}$ | 60–70 |
| 60 | $(6.0 \pm ND) \times 10^{-5}$ | 40–50 | NA | NA | NA | $(8.3 \pm ND) \times 10^{-6}$ | <10 |

a prelabeled cysteine is added to the protein of interest by protein trans-splicing (37, 38). Because all four inteins characterized here operate with a Ser+1 instead of a Cys+1 residue and are also free of Cys residues in the remainder of their IntC sequences (except for a Cys-2 in the gp41-8 IntC fragment), they would be ideally suited for this technology without the need for further mutagenesis.

Split inteins, like regular cis-inteins, can be genetically engineered to allow controllable in vitro N- or C-cleavage (21), which is useful for protein purification purposes, for example. A specific advantage of split inteins in this respect is that uncontrolled in vivo cleavage is circumvented, which may have caused poor yields of the target protein (39). We generated a C1A mutant for each of the four novel split inteins to test whether these proteins could be used for C-terminal cleavage applications. Previously reported naturally split inteins display very slow or no self-cleavage activity at all when mutated at the same position (26, 40). Ssp DnaE is the only reported natural split intein rendering a C-terminal cleavage rate of $1.9 \times 10^{-4}$ s$^{-1}$, but only after N-terminal cleavage has occurred. This can be induced by thiolyis, thus indicating a clear coordination of steps (12). Intriguingly, although the unprecedented high rate constants and efficiency of the four new inteins in the trans-splicing reaction might have suggested a highly optimized active site that is likewise resistant to aberrant side reactions induced by single amino acid mutations, we find that this is not the case. In contrast to the split DnaE alleles, our experimental data indicate that the naturally split inteins gp41-1$^{N(C1A)}$, gp41-8$^{N(C1A)}$, NrdJ-1$^{N(C1A)}$, and IMPDH-1$^{N(C1A)}$ exhibited C-terminal cleavage, although to different extents. Under conditions at pH 7 and 37 °C, this reaction was about 60–580%, 410%, and 110-fold slower than the trans-splicing reaction of the corresponding split inteins, respectively. Among the four inteins, gp41-1 displayed the highest C-cleavage reaction rate (Table 4) with a nearly complete conversion (85–90% yield). The NrdJ-1 and IMPDH-1 inteins exhibit similar constant rates and yields of C-terminal self-cleavage under the same conditions, whereas the gp41-8 intein shows the lowest rate and lowest yield of C-cleavage (near 50%).

It is fairly well known that modifications of native extein residues directly flanking the intein sequence can lead to inefficient trans-splicing or self-cleavage reactions (41). However, additional residues on the target protein are undesirable for some recombinant protein production applications, therefore making their removal essential. The current study shows that the four described new inteins retain their C-cleavage activity after deletion of their five C-terminal native extein residues, although at 37 °C, the C-terminal cleavage rates of the gp41-1, gp41-8, NrdJ-1, and IMPDH-1 inteins were decreased by 10–15, 5–3%, and 38.5-fold, respectively (Table 5 and Fig. 4E).

Removal of the native flanking residues also negatively affects, to differing degrees, the C-cleavage reaction yields of all split inteins except for the gp41-1 intein, which was still capable of driving the cleavage reaction to almost 90% precursor consumption.

The observed extraordinarily high constant rates of the novel inteins are intriguing and could not have been predicted before this study. The obvious prerequisites for this behavior are a highly optimized recognition and folding of the split intein fragments. For the association of naturally split intein fragments, complementary local charge distributions appear to be an important parameter (7, 27, 29, 42, 43). The fragments of the Ssp DnaE inteins are positively and negatively charged and form a complex in solution with a rate approaching the diffusion limit (29), whereas the rate-determining step appears to be an intramolecular rearrangement within the complex. Thus, although it was previously postulated that the gp41-1, gp41-8, NrdJ-1, and IMPDH-1 inteins have 10, 7, 7, and 3 interfragment salt-bridges, respectively, in the predicted interaction interface between the two split parts (27), it appears unlikely that the association could be faster than that observed for the Ssp DnaE intein or at least that this could account for an increased overall reaction rate. However, we propose that the high rates observed here are a consequence of the rapid folding into the active intein structure, the formation of highly optimized active sites at the N- and C-terminal junctions, and an optimal coupling of the individual steps during protein splicing.

We are currently unable to predict such a superior catalytic behavior from the primary sequence of the intein. Recent studies on the directed evolution of inteins indicated that a few amino acid substitutions can have a significant positive effect on their performance (30, 44–47). As previously described (27), the new split inteins are not closely related to other established split and full-length inteins. In fact, their protein sequences only share up to 40% similarity to other known cyanobacterial naturally split inteins (7, 35). Among them, the gp41-1, gp41-8, NrdJ-1, and IMPDH-1 inteins also share low identity (40–50%), denoting relevant sequence variations in their intein halves.
The sequences and gene organization of the reported split inteins are closely related to those found in phages and viruses, suggesting their viral origin (27). It is known that inteins and split inteins contain six conserved protein splicing motifs of the HINT (Hog/Intein) family: N1 to N4 present in the N-terminal fragment of the split intein and C1 and C2 present in the C-terminal split intein fragment (5–7, 48). Of the six conserved motifs, N1, N3, C1, and C2 are the most conserved ones among the naturally split inteins and have been described as block A, B, F and G, respectively. All these sequence motifs can be identified in the gp41-1, gp41-8, NrdJ, and IMPDH-1 inteins. The only apparent major difference to the Sp and Npu DnaE inteins is a histidine as the penultimate amino acid in the G box, which in the well characterized natively split cyanobacterial inteins is a serine (data not shown). However, in most other inteins, a His residue is also found at this position, which is postulated to assist in cyclization of the ultimate Asn residue to effect cleavage at the C-terminal splice junction (49). Thus, the new inteins characterized here exhibit canonical key catalytic residues and do not represent unusual features in their primary sequences.

Altogether these results show that the gp41-1 intein is superior on all accounts to those investigated in this study. It is very tolerant to a broad range of reaction conditions and tolerates changes in its splicing junction region for C-terminal cleavage reactions. Most importantly, when compared with all other split inteins reported so far, from natural, artificial, or engineered origin, the four inteins characterized in this study show the highest reaction rates and efficiencies in the trans-splicing reaction. In particular, the gp41-1 intein increases the limits of the fastest split intein ever described and holds great potential as a protein ligation tool or in self-cleavage processes for protein purification.

Acknowledgments—We thank all ERA BIOTECH® staff, especially Núria Martínez and Rosa Sellabona for validation experiments; Didac Marco for the intein sequence alignment analysis; and Imma Llop-Tous and Pau Marzábal for discussions and critical reading of the manuscript.

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