Analysis of errors in finished DNA sequences: the surfactin operon of *Bacillus subtilis* as an example

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Increased productivity in DNA sequencing would not be valid without a straightforward detection and estimation of errors in finished sequences. The sequence of the surfactin operon from *Bacillus subtilis* was obtained by two different groups and by chance we were also working on the same chromosome region. Taking advantage of this situation we report in this paper, the number and nature of errors found in the overlapping part of the DNA sequences obtained by the three laboratories. The coincidence of some of the errors with compression in sequence ladders and with secondary DNA structures as well as the detection of frameshift errors using computer programs, are demonstrated. Finally we discuss the definition of a new sequencing strategy that might minimize both the error rate and the cost of sequencing.

Keywords: *Bacillus subtilis*, genome sequencing, accuracy, error, surfactin

INTRODUCTION

Recent developments in current DNA sequencing methodologies have produced systems with high productive capacity that have made it possible to start sequencing entire genomes of up to 100 Mb (Wilson *et al.*, 1994). The large amount of data obtained has to be accurate enough to produce final sequences with a minimum number of errors. Indeed, errors in DNA sequences may jeopardize some aspects of their analysis, for instance (a) in the design of specific oligonucleotides, (b) in studies of natural variations, and (c) in the detection of ORFs. The nature and distribution of errors in an individual sequence (i.e. a reading from one sequencing reaction) have been extensively studied (Chen & Hunkapiller, 1992; Khurshid & Beck, 1993; Koop *et al.*, 1993; Sulston *et al.*, 1992). The total error rate was shown to depend on the methodology used and on the length of reading. It is almost constant (< 1%) for readings not exceeding 350 bases, but it then increases very steeply, reaching more than 25% for readings longer than 550

bases (Koop *et al.*, 1993). This high level of error at one extremity of the sequence may be troublesome when assembling sequences in a shotgun approach. Errors in finished sequences have not been studied so precisely, and only estimates of the error rate in DNA sequences have been made, in two databases. They vary from 0.03 to 0.3% in GenBank (Krawetz, 1989) to up to 3% in the EMBL sequence database (Kristensen *et al.*, 1992). This is too high a level of error to do any of the above-mentioned analyses. As a matter of fact, the accuracy of a sequence can be greatly improved when increasing the depth of coverage (Churchill & Waterman, 1992), but if the sequencing conditions are not changed, only the number of random errors will be reduced.

Before becoming participants in the *B. subtilis* genome sequencing project, we were subcontracted to the A. Danchin group (Institut Pasteur, Paris) and received a λ clone containing a 13.7 kb DNA fragment for sequencing. It appeared to correspond to a part of the surfactin operon, *sfrA*, located at 29° on the *B. subtilis* genetic map (Anagnostopoulos *et al.*, 1993). This locus has also been determined by two other groups (Fuma *et al.*, 1993; Cosmina *et al.*, 1993). The three sequences, obtained independently by the three groups, overlap by more than 10 kb and offer the opportunity to analyse sequencing errors in finished sequences. In this report we present the

Abbreviation: indel, insertion/deletion.

The EMBL accession number for the nucleotide sequence data reported in this paper is X72672.

Table 1. Description and features of the discrepancies found between the X72672 (1) and D13262 (2) sequences

Position*	Type	Compression†/ stem-loop‡	Sequence bearing error	Corrected sequence§
11060	Indel	-/-	2	aaac`agaa
11062		-/-	2	acagAaaaa
11655	Inversion	-/-	2	tcagCGccaa
12750	Indel	+ / + ^a	2	cggaCgccc
12756		+ / + ^a	2	ccgg`cgag
14134	Indel	+ / -	2	ctgc`cgct
14136		+ / -	2	gccgCtcac
14431	Point	-/-	2	gagaAcgaa
15246	Inversion	+ / +	2	cattGTcggc
15469	Inversion	- / +	2	agtcCGctgt
16662	Point	-/-	2	acatAccga
16703		+ / +	2	gataGgcgc
16738	Indel	-/-	2	gccgGatgc
16765		-¶ / +	2	atcgGacgc
17523	Point	+ / + ^b	2	agttCgcca
17536	Point	+ / + ^b	2	gcggCttat
17716	Point	+ / + ^c	2 (Fig. 2)	atttCgcgc
17719	Point	+ / + ^c	2 (Fig. 2)	tcgcGcctg
17928	Inversion	+ / + ^{**}	1	cgcaGctttg
18482	Inversion	-/-	1	cggaGcttt
18755	Indel	-/-	2	atatGcgac
18770		-/-	2	gttc`gcaa
18939		-/-	2	caaa`acgg
18946	Indel	-/-	2	ggtt`catc
19004		-/-	2	atac`aacg
19126	Indel	-/-	2	cttg`aaat
19156		-/-	2	tctt`gaaa
19241	Inversion	+ / -	2	cgaaGcttct
19933	Indel	+ / -	2	tagcCGgaga
19952		+ / -	2	cgggGgcgc
20001	Indel	+ / +	2	aagcGGTttgt

* Positions refer to the numbering of sequence D13262.

† A + sign indicates that compressions have been detected in our sequencing gels.

‡ The presence of a stem-loop structure is indicated with +. When the corresponding stem-loop overlaps the discrepancy, a + sign is used. Normally, the distance between a discrepancy and a stem-loop does not exceed 2 bases. Identical letters (a, b and c) indicate that discrepancies correspond to the same stem-loop.

§ Upper-case letters indicate the corrected bases. In the case of inversions and point errors, the first upper-case letter corresponds to the indicated position. For indels, bases in upper-case letters have been inserted after the indicated position and ` means that the base at the indicated position has been deleted.

|| Single events of insertion or deletion, involved in the same frameshift and preserving the reading frame, are grouped as indels.

¶ No compression detected in this area, but a polymerase pause has been observed in many clones, suggesting that this region is difficult to sequence due to the presence of secondary structures.

** Unclassified stem-loop (18th position with the STEMLOOP program) presenting, when enlarged with 6 bases, a stable structure.

interpreted as base inversion (i.e. CG in one sequence and GC in the other). Indels induce frameshifts, but no ORF interruption is observed, because each deletion (insertion) is always followed either by an insertion (deletion) or by two deletions (insertions) in the sequence. Single indel events involved in the same frameshift and preserving the

reading frame were grouped. Thus, we finally distinguished three types of discrepancies: (1) single base substitutions; (2) base inversions; and (3) indels (Table 1). The first two types corrupted only one or two bases (and one or two amino acids), but the indel type changed as many as 24 amino acids.

Fig. 2. Analysis of the discrepancies at positions 17716 and 17719. (a) Multi-alignments of the sequences (X72672, D13262 and X70356) in this region. Two different alignments are presented (depending on the position of deletions in X70356) and the discrepancies are shown in lower-case letters. (b) Stem-loop structure possibly formed by the sequence underlined in (a). (c) Autoradiographs showing the sequences obtained on both strands (1, 2) using 7-deaza-dGTP. Base order is indicated over the lanes and sequence readings are shown on the left (1) or right (2).

We searched for discrepancies between the X72672 and D13262 sequences and used the X70356 sequence, determined from another strain, to resolve the conflicts. As indicated in Table 1, we found six single base changes, five GC to CG inversions, one GT to TG inversion and nine indels within the 10253 bp corresponding to the overlap. After comparison with the X70356 sequence, 27 discrepancies (single event) were interpreted as sequencing errors in D13262 and two in X72672. Two other differences (17716 and 17719) cannot be resolved just by sequence alignment because a 2 bp deletion was observed in X70356 (Fig. 2). Depending on the location of the

deletion in the alignments, one or two errors could be assigned to X72672. In fact, we checked our data on both strands at these positions carefully (Fig. 2), and did not find any evidence of errors in our sequence (X72672). In the end, all discrepancies were resolved and we obtained a sequence of *B. subtilis* strain 168, which is most likely free of errors (Table 1).

In total we found 31 discrepancies. Among them, 16 correspond to areas difficult to sequence and, in most cases, to compressions detectable in our gels (compressions correspond to abnormal mobilities of DNA fragments in sequence ladders). In one instance we found polymerase pauses in almost all the templates we sequenced, suggesting that some local properties of the DNA affect the enzyme.

Secondary structures and frameshifts

It has been proposed that compressions might be caused by stretches of secondary structure in single-stranded DNA, such as stem-loops, that are stable even in the denaturing environment of a sequencing gel (Sambrook *et al.*, 1989). We used the STEMLOOP program of the GCG package (Devereux *et al.*, 1984) to search for inverted repeats in the overlapping DNA fragments. We set the parameters to a minimum stem length of 5 bp and a loop size between 2 and 5 bp. Among the 15 more stable stem-loops found over the 10253 bp, only seven are shared by both sequences (X72672 and D13262). The other eight hairpins are specific to X72672 and are associated with 11 discrepancies that correspond to compression artifacts in our gels (Table 1). They were all resolved and in 10 instances, X72672 appeared to be the correct sequence. This correlation between discrepancy, stable stem-loop and compression artifact, suggests that if a discrepancy is observed between two sequences at the level of a compression, the sequence giving the more stable stem-loop has the best chance to be the correct one. As an illustration, the unresolved discrepancies (17716, 17719), which were found to be associated with a stable stem-loop only in X72672, have been resolved as sequencing errors in D13262 (Fig. 2).

Therefore, searching for secondary structures can be used to resolve discrepancies between two sequences. One can also look for frameshift errors. This approach is particularly useful when only one sequence is available.

The distribution of the non-overlapping trinucleotides differs for each frame in coding regions and this property could be used for detecting frameshift errors. We tested this assumption by using a new algorithm based on an approach similar to the one developed in the RECSTA program (Fichant & Gauthier, 1987) which was an algorithm devoted to the prediction of coding and non-coding sequences. We computed the trinucleotide frequencies of each frame and used the correspondence analysis method to obtain linear combinations of the trinucleotides that gave the best discrimination between the three frames. For prediction, a window was shifted along the sequence with a 30-base step to keep in frame with the ORF. A frameshift error is detected when the

Fig. 3. Frameshift errors predicted with our program in the overlapping region. In this region (from 10283 to 20535), the first reading frame corresponds to the coding frame. A 90 bp window was shifted along the sequence with a 30 bp step, and assigned by the program to one of the three reading frames. Only two regions are predicted in frame 2, and both have been described as frameshift errors of more than 60 bp (positions 16703–16765 and 18939–19004 in Table 1).

frame of the window does not correspond to the frame of the ORF. Using a 90-base window, which corresponds to the best ratio between error predictions and false predictions, two regions of D13262 that should be in frame 1 were assigned to frame 2 (16703–16765 and 18939–19004, Fig. 3). They were predicted as frameshift errors and they correspond to the larger discrepancies involving 61 and 64 bp (see grouped indels, Table 1).

We shall not describe in detail the analysis of the discrepancies observed in X70356. The sequence was obtained from a *B. subtilis* strain that produces surfactin (strain 168 does not), and at least some of the discrepancies might be due to polymorphism. However, two regions seem to correspond to sequencing errors: the duplicated area (bases 16329–16345, duplicated after position 16346) and a region where almost all Gs (16/17) are replaced with Cs (from base 10989 to base 11042). When running the modified RECSTA program with the same parameters as above, this area is predicted as a frameshift error, suggesting that the G to C exchanges correspond to sequencing errors. It should be pointed out that the program also predicts an error in X70356, outside the overlapping region, in position 3769–3829. It corresponds to a frameshift in this sequence. Therefore, all the frameshifts observed in X70356 and D13262, involving a large number of bases (about 60), were detected by the method with no false positive. These preliminary results suggest that statistical methods can be used to check new sequences for frameshift errors. This kind of error is not the most frequent (3.5% of the 86 discrepancies found between the three sequences) but certainly it is the most bothersome as it corrupts a large stretch of the amino acid sequence.

DISCUSSION

Two sequence fragments determined in the same *B. subtilis* strain (168, *trpC2*) overlap over more than 10 kb (this work and Fuma *et al.*, 1993). Twenty-nine discrepancies were observed between the two sequences. A third sequence obtained from another strain (Cosmina *et al.*, 1993) was used to resolve the conflicts and allowed the estimation of the total error rate in the finished sequences, corresponding to 0.02% for our sequence (X72672) and

0.27% for D13262 (Fuma *et al.*, 1993); these numbers included random and systematic errors. The latter kind of error, corresponding to sequence-specific discrepancies associated with compressions on our gels, can be discarded. Thus, the estimates of the random error rate for X72672 and D13262 are 0.01% and 0.13%, respectively.

According to the theoretical work of Churchill & Waterman (1992), the 0.13% random error rate, obtained for D13262, is in the range of what is expected for a shotgun approach (assuming a 10^{-2} error rate for gel reading and a redundancy rate of 6). On the other hand, the 0.01% random error rate, which we obtained with a coverage of 5.5, is one order of magnitude below this standard. Manual film readings have been shown to give the lowest error rate (5×10^{-3} ; Khurshid & Beck, 1993) but it seems that ours were done with a much higher degree of accuracy, very likely reaching a reading error rate much lower than 10^{-3} . Indeed, we used a manual reader (see Methods) equipped with a speaker that echoes back bases when entering them, thus allowing us to easily detect misreadings. In addition, almost all the sequences were read independently by two investigators.

Only four discrepancies in X70356, corresponding either to true errors or to natural variations, are associated with compressions detected in our gels. This is a small value in comparison to the 15 discrepancies found in D13262 also shown to correspond to compressions in our gels. This difference is most likely due to the sequencing method used by the Japanese team (Fuma *et al.*, 1993). They certainly must have had difficulties detecting compressions, and using a 'mono-protocol' approach, without any change in the sequencing reactions or electrophoresis, they were unable to resolve them. Thus, when assembling sequences in a shotgun approach, systematic false readings of the same area in different templates may result in misconstruction of the finished sequence, giving errors in this region. Tools to resolve the compressions are available, but the limiting factor is their detection. A manual reading of gels by an expert gives better results than automatic film or fluorescence readers, but at a much higher cost. Thus, compressions should be well characterized in such a way that defined rules could be included in algorithms to achieve automatic detection. On the other hand, as systematic errors are assumed to be sequence-specific, one can expect to be able to detect such error-prone regions throughout the analysis of the local sequence properties. Characterization of their folding tendency is the first step along this route. To complete this approach, a database of sequencing data (with regard to compressions and their readings) would be very useful.

DNA sequencing is still a very expensive technique and one way to reduce its cost would be to decrease the redundancy rate. We have recently proposed a new strategy to achieve this goal with large fragments (> 100 kb, Denizot & Guenoche, unpublished data). Computer simulations showed that all contigs can be ordered with a mean coverage between 2 and 3. The gaps between contigs are easily determined by a directed approach. Each base is sequenced on both strands and areas

showing any sequence discrepancies are sequenced once again. In the case of a pure random error, with an error reading rate (r) of 10^{-2} , the likelihood of obtaining an error at the same position in the third sequence is given by $r^2 = 10^{-4}$. An on-line analysis of the assembled sequence with the modified RECSTA program will be helpful in detecting frameshift errors. Since less work is needed to cover rapidly the total DNA fragment and to obtain the sequence roughly at a low random error rate, more effort could be made to detect and resolve systematic errors to obtain a very accurate finished sequence.

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