JOURNAL OF BACTERIOLOGY, Mar. 1989, p. 1602–1608
0021-9193/89/031602-07$02.00/0

Vol. 171, No. 3

Amplification of Bacterial Genomic DNA by the Polymerase Chain Reaction and Direct Sequencing after Asymmetric Amplification: Application to the Study of Periplasmic Permeases

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Received 3 October 1988/Accepted 20 December 1988

The polymerase chain reaction (PCR) has been used to amplify DNA fragments by using eucaryotic genomic DNA as a template. We show that bacterial genomic DNA can be used as a template for PCR amplification. We demonstrate that DNA fragments at least as large as 4,400 base pairs can be amplified with fidelity and that the amplified DNA can be used as a substrate for most operations involving DNA. We discuss problems inherent in the direct sequencing of the amplified product, one of the important exploitations of this methodology. We have solved the problems by developing an "asymmetric amplification" method in which one of the oligonucleotide primers is used in limiting amounts, thus allowing the accumulation of single-stranded copies of only one of the DNA strands. As an illustration of the use of PCR in bacteria, we have amplified, sequenced, and subcloned several DNA fragments carrying mutations in genes of the histidine permease operon. These mutations are part of a preliminary approach to studying protein-protein interactions in transport, and their nature is discussed.

One of the most common situations in microbiological research is the isolation of mutants with particular properties and their characterization at a variety of levels, the identification of the mutation at the nucleic acid level being among the most important. To sequence interesting mutations, it has been necessary to transfer them to plasmids specially designed for the purpose and already containing the gene of interest, by using genetic selections that allow recognition of the mutation-containing clones. Alternatively, the mutated genes could be cloned anew from the chromosome and the clones could be identified either by genetic selection or by Southern hybridization with wild-type material. These approaches are relatively cumbersome, especially for rapid analysis of large numbers of mutants; some of them (such as genetic selections operating on the cloned material) often are not feasible. We describe here the application of the polymerase chain reaction (PCR) to solving this problem.

The PCR has been used successfully to amplify specific regions of genomic DNA from a variety of eucaryotic systems (12, 15, 17). Clearly, it is essential to determine whether this technique is applicable to bacterial genomic DNA. The PCR allows the selective amplification of DNA fragments up to several kilobases in length, as long as the sequence of the two extremities of the region of interest is known. In brief, oligonucleotides bracketing the DNA segment of interest and hybridizing to opposite strands of the target sequence are used as primers for elongation with DNA polymerase. Extension of each oligonucleotide primer while hybridized to the original template yields single-stranded DNA copies of indefinite length which increase linearly in number. These single-stranded DNA copies act as templates for further new rounds of hybridization to and elongation of the primers, yielding single-stranded DNA copies of a specific length (bracketed by the two primers) which increase exponentially in number. Thus, repeated cycles of heat denaturation, annealing, and elongation result in an exponential increase in the number of copies of the target DNA (12). The amplified material thus produced is suitable for a variety of subsequent operations.

We have used PCR to amplify specific regions of chromosomal DNA from both *Escherichia coli* and *Salmonella typhimurium*. We present general strategies for sequencing, cloning, and transferring mutations of interest into plasmid vectors. In particular, as an illustration of our use of this methodology, we present the initial characterization of mutants affecting protein domains responsible for protein-protein interactions within the histidine permease complex (1).

The histidine permease is composed of four proteins: a soluble receptor (the periplasmic histidine-binding protein, HisJ) and three membrane-bound proteins (HisQ, HisM, and HisP). In our present model for transport, the liganded receptor (HisJ) undergoes a conformational change which allows its interaction with one or more of the components of the membrane-bound complex. This interaction signals the membrane-bound complex to undergo additional conformational changes that result in the release and passage of the substrate through the membrane (1). The coupling of energy to this process is likely to be mediated by the HisP protein, which binds ATP (8; A. K. Joshi, S. Ahmed, and G. F.-L. Ames, J. Biol. Chem., in press). We have shown genetically that HisJ is organized into two domains, one responsible for binding the substrate and the other for interacting with the membrane-bound complex (4). Genetic techniques suggested that HisJ interacts with the peripheral membrane-bound HisP protein (2); biochemical methods, using formaldehyde and photoactivatable cross-linking techniques, have clearly shown that HisJ interacts with the membrane-bound HisQ protein (15a). Localization studies on isolated membranes indicate that portions of HisP are located on the inner surface of the cytoplasmic membrane (R. Kerppola and G. F.-L. Ames, unpublished results). This raises the question of what form the HisJ-HisP protein interaction takes. Does it occur through direct protein-protein contact within the membrane, or is it indirect, presumably mediated through HisQ and HisM? One approach towards understanding this complex pattern of protein-protein interactions involves the

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isolation of numerous mutants in which such interactions are altered and the identification of the amino acid residues involved. With the availability of PCR, this approach has become feasible, since it is possible to sequence rapidly a large number of mutations. Here we present the sequence of a few interaction mutations as an illustration of the applicability of the PCR method to the problem of mutant characterization. We also give an example of its use to obtain mutant genes under the control of a specific promoter. Finally, we introduce a new method for the asymmetric amplification of only one strand of DNA, which allows the rapid direct sequencing of the amplified material.

MATERIALS AND METHODS

Materials. *Thermus aquaticus* (*Taq*) DNA polymerase was purchased from Perkin Elmer-Cetus; deoxynucleoside triphosphates were purchased from Pharmacia; Random Primer kit and M13mp18 RF were purchased from Boehringer; Zeta-Probe was purchased from Bio-Rad Laboratories; restriction enzymes were purchased from Boehringer and New England BioLabs, Inc.; Sephacryl S-300 was purchased from Sigma Chemical Co.; Sequenase sequencing kit was purchased from US Biologicals.

Bacterial strains. LT2 is the wild-type *S. typhimurium* strain. All mutant strains carried a promoter up mutation in the transport operon (*dhuA1*) and (except for TA300) a deletion of the histidine biosynthetic gene (Δ*hisF645*). Additional mutations were as follows: TA271, none; TA300, *hisJ5625*; TA5096, *hisP9083*; TA5095, *hisP9016*; TA347, *hisP5700*; TA3258, Δ*hisP8963*. TA4211 was the *E. coli* K-12 strain used.

Amplification of bacterial chromosomal DNA by PCR. Chromosomal DNA was prepared by the method of Ardeshir et al. (3). The PCR reaction (17) was carried out in a 100-μl final volume containing 1 μg of chromosomal DNA, 67 mM Tris chloride (pH 8.8, at 25°C), 6.7 mM magnesium chloride, 1.6 mM ammonium sulfate, 10% dimethyl sulfoxide, 10 mM β-mercaptoethanol, 6.7 μM EDTA, a 1.0 mM concentration of each deoxynucleoside triphosphates, 1.0 μM oligomers, and 1 U of *T. aquaticus* DNA polymerase. The amplification reaction was carried out for 30 cycles in the DNA Thermal Cycler (Perkin Elmer-Cetus). The DNA was denatured at 93°C for 1 min, annealed at 55°C for 1 min, and extended at 70°C. The extension period depended on the size of the amplified fragment: 2 min for 180 base pairs (bp), 3 min for 1,000 bp, and 5 min for 4,400 bp. To analyze the amplification products, 2 μl of the reaction mix was electrophoresed on 1% agarose visualized with ethidium bromide, and, if necessary, transferred to Zeta-Probe for Southern analysis as described by Reed and Mann (16).

Asymmetric amplification of single-stranded DNA for direct sequencing of amplified product. Amplification for direct sequencing was carried out with a limiting concentration of one of the primers in order to get preferential amplification of one strand. The reaction was carried out as for the standard PCR as described above, with the following modifications. The final concentration of the limiting primer was 0.012 μM (1.2 pmol) and of the excess primer was 0.6 μM (60 pmol). Following 40 cycles of amplification, 4 μl was electrophoresed on 1% agarose and the amplification product was visualized with ethidium bromide. The amplification reaction mix from the limiting primer reaction was passed through a spin column of Sephacryl S-300 to remove salts, deoxynucleoside triphosphates, and primers. The sample was alcohol precipitated and sequenced by using the Sequenase sequencing kit, with the following modifications: the labeling mix was diluted 1:100 instead of 1:5; the labeling time was about 15 s; and the termination reaction was stopped after 1 min.

Cloning of amplified DNA into plasmid. A portion of the *hisJ* gene was amplified from chromosomal DNA from TA300 by using primers J7 and J6. The 951-bp amplified DNA was then digested with *Hin*dIII and *Sca*I. The 431-bp fragment expected to have the mutation was purified and ligated into the purified 3.3-kilobase-pair fragment obtained by digestion of pFA54 with *Hin*dIII and *Sca*I. This resulted in the replacement of the corresponding wild-type 431-bp *hisJ* DNA fragment present in plasmid pFA54. This plasmid carries the *hisJ* gene under control of the *tac* promoter. The presence of the mutation in the resulting plasmid pFA89 was ascertained by sequencing. pFA54 and pFA89 plasmid DNAs were purified on a CsCl gradient and sequenced with Sequenase by using oligomers J2 and J3 (which hybridize at sites 298 and 555, respectively) as sequencing primers. Sequencing by the Maxam-Gilbert technique and cloning of amplified DNA into M13 vectors for sequencing were performed by established procedures (11, 18).

RESULTS

Amplification from chromosomal DNA. Chromosomal DNA from *S. typhimurium* was used as a source of amplifiable material. Figure 1A (lanes 1 and 2) shows that *S. typhimurium* chromosomal DNA, from either the wild type or a permease point mutant, can be successfully used as template in the amplification of *hisP* DNA by using primers P6 and P7 (Fig. 1B). A unique product of the expected size (938 bp) is formed. To ascertain that this fragment was from within the *hisP* gene, DNA from Δ*hisP8963*, which has a 96-bp *hisP* deletion located between P6 and P7, was amplified; the amplified product, accordingly, was 96 bp smaller (Fig. 1A, lane 3). The specificity of the amplification procedure was confirmed by Southern hybridization of the amplified DNA fragments to an authentic *hisP* gene probe (lanes 4 to 6). Fragments at least as large as 4,400 bp could be selectively amplified by this method (data not shown). The general applicability of this technique was shown by amplifying several other genes from the *Salmonella* chromosome: *hisJ* (by using J7 and J6 in lanes 7 and 8; confirmed by Southern hybridization as shown in lanes 9 and 10), *hisG*, *argT*, *hisQ*, and *hisM* (data not shown). The amplified DNA was, in all cases, a unique product of the expected size. Often, it is useful to synthesize fragments that contain restriction sites at one or both ends, since this greatly facilitates sequencing by various techniques and in vitro cloning. This can be easily achieved by using primers with tailored restriction sites. Examples of the use of such primers appear in Fig. 1A, lanes 1 to 6. Primers P6 (with *Bgl*II) and P7 (with *Ava*I), including as much as 30% nonhomology (9 out of 31 nucleotides in P6) at the 5′ end, still yield a unique product. In addition, because of the extensive homology between *S. typhimurium* and *E. coli*, we were also able to amplify *hisP* DNA from the *E. coli* chromosome by using primers that are homologous to *S. typhimurium* DNA (data not shown).

Sequencing of amplified DNA by standard techniques. One of the main advantages of PCR is that it provides the means of synthesizing DNA fragments in vitro for sequence analysis of mutants without the need to subclone the mutated gene. Our main objective has been to develop a satisfactory sequencing technique involving minimum manipulations of the amplified material. We have investigated a number of different techniques to sequence the amplified fragments.

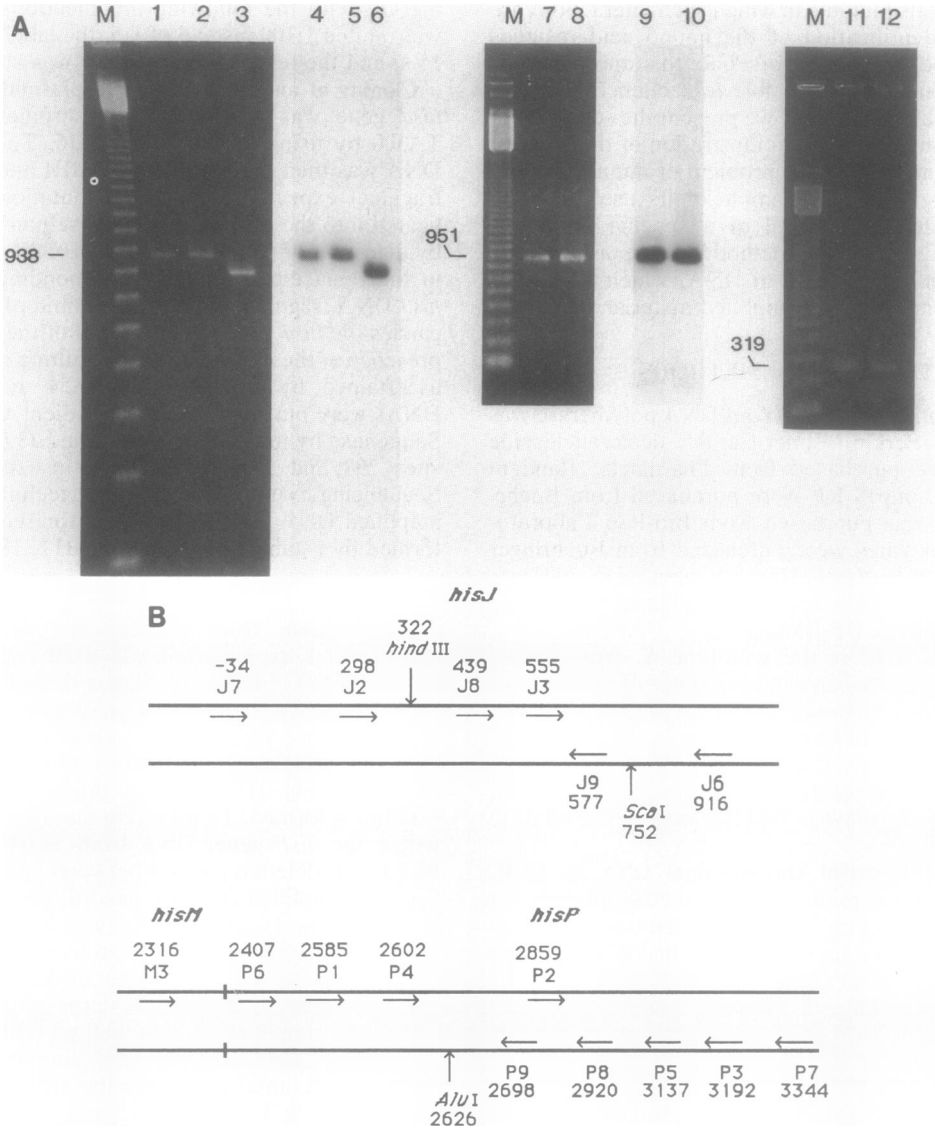

FIG. 1. Amplification of *S. typhimurium* chromosomal DNA by PCR. (A) Amplification of *hisP* and *hisJ* DNA. Chromosomal DNA was amplified with primers P6-P7 (lanes 1 to 6), J7-J6 (lanes 7 to 10), and P6 excess-P9 limiting (lanes 11 and 12). Genomic DNA was derived from TA271 (wild type for transport operon) (lanes 1, 4, 7, 9, and 11), TA347 (*hisP5700*) (lanes 2 and 5), TA3258 (Δ*hisP8963*) (lanes 3 and 6), TA300 (*hisJ5625*) (lanes 8 and 10), and TA5096 (*hisP9083*) (lane 12). Lanes 4, 5, and 6 are from a Southern analysis with a *hisP* probe; lanes 9 and 10 are from a Southern analysis with a *hisJ* probe. Lanes M contain a 123-bp ladder marker (Bethesda Research Laboratories). The *hisP* DNA probe was prepared by digesting pFA13 with *Pst*I and *Ava*I. The *hisJ* DNA probe was prepared by digesting pFA54 with *Hin*dIII. The digested DNA was purified on agarose gel and the appropriate fragments were labeled by using the random priming kit. (B) Diagrammatic representation of the *S. typhimurium hisJ* and *hisP* genes with locations of oligomers and relevant restriction sites. Numbering is as in reference 5. The following synthetic oligonucleotides were used (all sequences are 5′ to 3′; their location within the gene indicated is numbered according to reference 5; T [top] and B [bottom] indicate which strand the oligonucleotide sequences correspond to): P1, GCGCGACAAA GAT (T); P2, GCGCGCGCGCTGGCG (T); P3, GATGCGCCGCCGGGC (B); P4, AGCTCAAAGTGGCGGATAAA (T); P5, CAGC GAGCCTTTCAGGAATT (B); P6, TATAGATCT/AACGCTACGGCGGTCATGAAGT (T); P7, TATCTCGAG/ATTCCGGCGACGGC AATTCGCTTAT (B); P8, TATC/TCGAGCGCCGATGTGGGTTCAT (B); P9, TATCTCG/AGCACCGTCATGTGGCTCCAG (B); J2, CAGCAGGAAATCGCT (T); J3, CGCGCCAGCGAAGG (T); J6, TTGCCTCACATCACGCCGGAT (B); J7, GATCTCTTGGAAAGA-CAGTCTGTT (T); J8, TTCGGCAATGAGCACT (T); J9, TATCTCGA/GTAAGGAAACCTTGGCT (B); M3, GGGCGGAAAGACGCTGG (T). The nucleotides to the left of the slash are absent in the gene sequence and have been introduced into the primer to create a restriction site at the 5′ end: *Bgl*II (T) and *Ava*I (B); in addition, they also have an overhang of three nucleotides (TAT) at the 5′ end to facilitate enzyme binding and restriction.

Direct sequencing of amplified, unpurified fragments can be performed by the Maxam and Gilbert technique. Both 5′ ends of the amplified fragments (which are unesterified) are end labeled, followed by removal of one of the labeled ends by restriction digestion, thus obviating the need for strand separation prior to sequencing. In the absence of a satisfactory enzyme site at a suitable position in the amplified fragment, we have used primers with tailored restriction sites to remove preferentially one of the labeled 5′ ends (e.g., P6, carrying *Bgl*II). The sequence of selected regions, amplified by using P4-P5 primers and removing one labeled end by digestion with *Alu*I, from strains LT2 and TA271 was

found by this method to be identical to the published sequence (5 and data not shown). However, this sequencing procedure is somewhat laborious and not suitable for analysis of a large number of mutants.

The chain termination-sequencing procedure of Sanger and Coulson (18) is very convenient and rapid and can be performed on circular and double-stranded DNA (9). Direct sequencing of linear amplified, unpurified DNA by this technique has been attempted earlier (7, 21, 22). However, with this method the maximum readable sequence was only about 100 bp, and the size of the amplified fragment was limited to 220 bp (7). In addition, only ^{32}P-labeled oligomers, but not ^{35}S-substituted deoxynucleoside triphosphates, could be used. Our initial attempts to sequence 536 bp of amplified DNA (a *hisP* P4-P5 fragment) by this procedure were not successful, because of the frequent appearance of bands running through all four lanes, presumably because of termination of elongation (data not shown). This problem appears to be at the point of keeping the longer DNA strands (>220 bp) apart during elongation. Addition of single-strand-binding protein during sequencing, performing the sequencing reaction at high temperatures up to 50°C, and using reverse transcriptase did not solve the problem.

Sequencing of amplified products can be done by first subcloning the amplified, unpurified DNA into M13 vectors (19). P6 and P7 primers carrying restriction enzyme sites were used for amplification. Following cloning of the P6-P7 product into M13mp18, we have been able to sequence rapidly the entire P gene (about 0.9 kilobase pairs) by using multiple primers (data not shown). As expected, this offers all the advantages of an M13 sequencing system. However, because of the lack of absolute fidelity of the polymerase, errors due to misincorporated bases are perpetuated through repeated copying cycles. Since cloning into M13 singles out individual single-stranded DNA copies, it is possible that the particular bacteriophage clone selected for sequencing is derived from a copy containing one of these errors. The earlier an error occurs in the amplification process, the higher its frequency is in the final population (17). Therefore, this is an important drawback of this method. To overcome this problem it is necessary, and sufficient, to sequence three separate transformants for each product. While this sequencing approach is satisfactory, the need to sequence multiple clones and the time required to subclone the amplified DNA into M13 vectors is a major disadvantage.

Sequencing of asymmetrically amplified fragments. The problems emerging during the direct chain termination-sequencing attempts strongly indicate that it should be possible to sequence directly an amplified product as long as it is single stranded. By limiting the concentration of one of the two primers it is possible to accumulate preferentially single-stranded DNA by elongation from the primer present in excess. A ratio of primers of 1:50, replacing the usual ratio of 1:1, results in the synthesis of both a double-stranded fragment and of single-stranded DNA. Figure 1A (lanes 11 and 12) shows the DNA obtained from such a reaction: a fragment of the correct size is formed presumably by elongation of both primers. The single-stranded product can be visualized either by ethidium bromide staining, if it is sufficiently abundant, or by Southern hybridization with an appropriate probe. Southern hybridization of lanes 11 and 12 showed the presence of a band moving slightly faster than the double-stranded fragment (data not shown). Similar results were obtained in a reciprocal experiment using limiting concentrations of the other primer (data not shown). An autoradiogram of a Sanger sequencing gel performed on DNA thus amplified is shown in Fig. 2, for both wild-type and mutant DNA. The sequence change responsible for mutation *hisP9083* can be easily discerned. This technique has been used successfully to sequence fragments up to 500 bp (data not shown). Compared with the other techniques discussed, it is far less cumbersome and far less time-consuming. Of the methods utilized in the present study, this is the method of choice for screening a large number of mutants (Table 1). While this paper was in preparation, an independent publication describing this methodology and its application to a eucaryotic system was submitted for publication (3a).

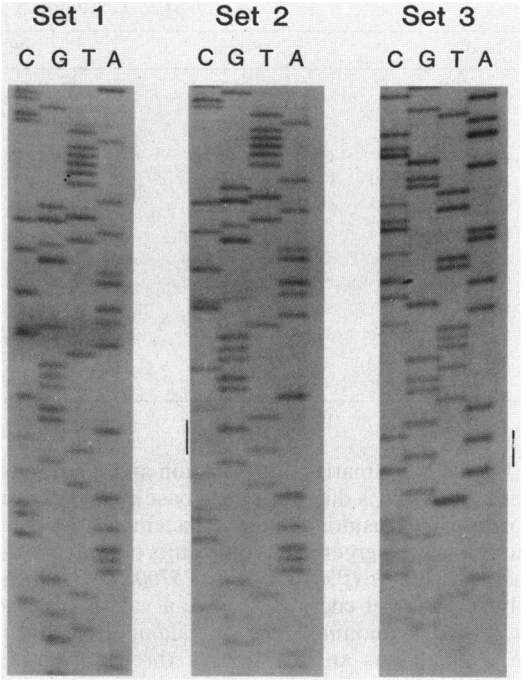

FIG. 2. Direct sequencing of DNA amplified by asymmetric amplification. The genomic DNA was TA271 (*hisP*$^+$) amplified with P6 excess-P9 limiting (Set 1); TA5096 (*hisP9083*) amplified with P6 excess-P9 limiting (Set 2); and TA5096 (*hisP9083*) amplified with P8 excess-P4 limiting (Set 3). Amplified DNA for sets 1 and 2 was from the same reaction mixture as in Fig. 1A, lanes 11 and 12, respectively. Vertical bars indicate mutation sites.

Analysis of histidine permease mutants involved in protein-protein interaction. With a view to understanding the molecular mechanism of periplasmic permeases, we have utilized PCR to identify the nature of several histidine transport mutants causing alterations in protein domains presumed to be involved in protein-protein interactions between the receptor, HisJ, and the membrane-bound complex. The following mutations were chosen for amplification and sequencing. *hisJ5625* causes a transport defect that results in a defective interaction of HisJ with HisP, leaving the histidine-binding site of HisJ intact (10). Suppressor mutants were isolated that correct the transport defect of *hisJ5625* (2 and unpublished data). Several of these mutations were shown to map in the *hisP* gene. In the selection of the suppressor mutants, it was expected that since HisJ interacts with HisQ (20), *hisQ* suppressor mutants should arise. However, in our initial screening, no *hisQ* mutants were obtained. The sequences of three *hisP* suppressor mutants, *hisP5700*, *hisP9016*, and *hisP9083*, together with that of *hisJ5625*, were obtained by a combination of the three methods described

TABLE 1. Summary of PCR product sequencing techniques

Method	Strain	PCR primers	Sequencing primer(s)	No. of base pairs read
Maxam and Gilbert	TA271	P4-P5		200
	LT2	P4-P5		200
M13 cloning	*hisP5700*	P6-P7	P5, P7, P8	900
	hisP9016	P6-P7	P5, P7, P8	900
Plasmid sequencing	pFA54		J2, J3	400
	pFA89	J6-J7	J2, J3	400
Direct sequencing	*hisJ5625*	J8 excess-J9 limiting	J9	125
	hisP8963	P5 excess-P2 limiting	P2	150
	hisP9083	P6 excess-P9 limiting	P9	208
	hisP9083	P8 excess-P4 limiting	P4	212

above. Figure 3 summarizes the location and nature of these mutations. *hisJ5625* is due to a single base change, from C to T, at position 526, resulting in the replacement of arginine by cysteine. This is in agreement with results obtained by amino acid analysis (13). *hisP9016* and *hisP5700* are located near the carboxy-terminal end and replace a valine and a threonine with a methionine and an alanine, respectively. *hisP9083*, which has an insertion of three nucleotides, is located in the central region of *hisP* and results in the insertion of a threonine. We will discuss possible interpretations of the nature of these mutations (see below).

Use of amplified DNA for cloning into vectors. A frequent need arises to subclone mutant genes for several purposes into appropriate vectors. For example, it is often necessary to investigate the phenotype of a particular mutation in a variety of backgrounds. As a rapid alternative to subcloning anew the mutated gene, PCR offers a simple solution, by allowing the introduction of amplified genes, or fragments thereof, into vectors already containing the gene under study. This procedure involves replacing an appropriate portion of a cloned gene with fragments amplified from chromosomal DNA carrying the mutation of interest. Here we describe the application of this method to the construction of a plasmid that overexpresses a HisJ mutant protein, the product of *hisJ5625* described above. A 951-bp fragment of the *hisJ5625* gene was amplified and used as described in Materials and Methods to replace in vitro the corresponding region of the wild-type *hisJ* gene under the control of the *tac* promoter in pFA54. The sequence of the entire *hisJ* gene on the newly derived plasmid, pFA89, showed a single base change from C to T at position 526, with no undesired misincorporations. pFA89 was shown to express the mutant HisJ protein in the same quantities as the wild type and to exhibit all the known biochemical properties of the mutant HisJ protein (data not shown). The overproduction of the altered HisJ protein is currently being exploited for crystallization purposes in order to define the domains that are involved in interacting with the membrane components (C.-H. Kang, S. H. Kim, K. Nikaido, and G. F.-L. Ames, unpublished data).

DISCUSSION

PCR provides a versatile tool for rapid genetic analysis. Over the past few years it has been used very extensively in eucaryotic systems. However, applications of this methodology to the study of procaryotic systems have not yet appeared in the literature. One of the basic requirements for specific amplification of a region within the chromosome is the sequence specificity of the oligonucleotide primers. Both the nucleotide composition and the length of the primers were found to be critical for imparting specificity of amplification. Use of GC-rich oligomers consistently gave multiple products. For example, P1 and P3 primers with A+T to G+C ratios of 6:11 and 2:13, respectively, gave several larger and smaller products, in addition to the expected 608-bp *hisP* product (data not shown). The requirements for specific amplification appear to be an equal ratio of A+T to G+C, with no contiguous stretch of any individual nucleotide; homology between the two primers should be minimal, to deter hybridization of the primers with each other. In addition, oligomers of at least 20 bp were found to be more specific than shorter oligomers.

An important aspect of PCR is the ability to sequence rapidly the products of amplification to ascertain both the nature of the template DNA and the presence of copying errors. The Maxam-Gilbert chemical sequencing method was used during the initial stages, since all attempts at Sanger sequencing of the double-stranded linear DNA were unsuccessful. Since Maxam-Gilbert sequencing is via bulk

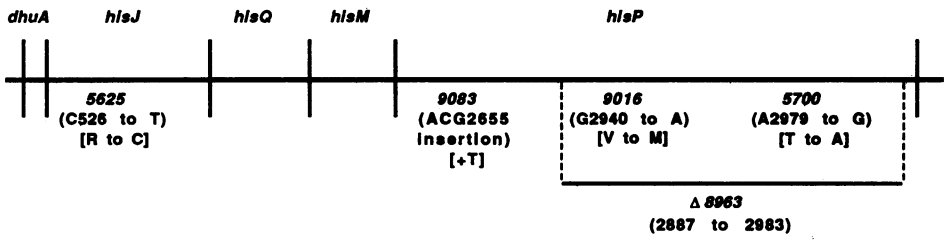

FIG. 3. Diagrammatic representation of mutations in the *S. typhimurium* histidine transport operon. The horizontal line represents the chromosome and is not to scale. Mutation numbers are in italics; the nucleotide positions where the changes have occurred are in parentheses; the resulting amino acid changes are in brackets.

product analysis, it is insensitive to misincorporation errors, unless the misincorporation occurs during the early cycles of PCR (17). Using this method, we did not find any errors in a total of 800 bp sequenced from six mutant strains. However, the inherent drawbacks of this procedure (the large number of manipulations, the use of several toxic reagents, and the fuzziness of the sequencing gel bands due to the ^{32}P) led us to try other methods to sequence the amplified products. Cloning into M13 vectors the amplified products obtained from primers with tailored enzyme sites provides a very useful substrate for subsequent dideoxy-sequencing reactions. As explained in Results, the ambiguities resulting from misincorporations and the time required for cloning into M13 vectors are the main drawbacks of this technique. The frequency of misincorporation errors, expressed as a percentage of the number of base pairs sequenced, is 0.07% under our conditions (3 errors out of 4,351 bp sequenced). This is considerably lower than the 0.25% reported by Saiki et al. (17) and may be due to the different lengths of our amplified products; our product was 1,000 bp, as opposed to 239 bp in the analysis by Saiki et al.

Since the solution to direct sequencing appeared to be at the level of obtaining single-stranded DNA, attempts were made to obtain amplified single-stranded DNA. Use of only one oligomer for the PCR reaction failed to give sufficient DNA for sequencing (data not shown). This is probably due to the fact that a single oligomer would result in a linear increase in product, unlike the situation with two oligomers, in which the increase in product is exponential. Therefore, we used a small concentration of a second oligomer which initially would permit synthesis of both strands, thus providing a large quantity of substrate for further single-strand synthesis during later cycles of amplification. This modification results in enough single-stranded DNA for direct sequencing. An important advantage is that misincorporation errors would not be picked up because of bulk product sequencing. This technique also permits sequencing both strands by carrying out two amplification reactions, each with either oligomer in limiting amounts. This has been the most satisfactory method of sequencing for amplification products of sequences up to 500 bp in length. Larger fragments could not be amplified by this method, possibly because insufficient quantities of single-stranded DNA are produced during amplification. It is likely that the two DNA strands in large fragments anneal to each other very efficiently, thus competing with the limiting primer. At present, M13 cloning is the only feasible technique for sequencing fragments larger than 500 bp.

The specificity of the primers plays a more important role in the single-strand asymmetric enrichment reaction than in the regular amplification reaction, where they are present in equimolar amounts. For example, M3-P9 oligomers gave a unique product of 383 bp in the regular reaction. The P9 excess-M3 limiting reaction yielded two bands. One of the bands was the double-stranded product, while the other possibly was the single-stranded product of P9 elongation. However, when M3 was used in excess with P9 limiting, multiple products were obtained, indicating that under these conditions M3 was being less specific (data not shown). Also, when a less specific oligomer is used in excess, it binds to different locations on both strands, frequently resulting in double-stranded products originating from a single oligomer (data not shown). However, since either the limiting oligomer or an internal oligomer is used as sequencing primer, these alternative products do not create problems.

The particular mutants we have chosen to illustrate the usefulness of the PCR technique in bacteria are part of our ongoing project on the molecular mechanism of protein-protein interaction in periplasmic transport systems. The *hisJ5625* mutation is located in a region of HisJ that is strongly conserved in another periplasmic protein, LAO, that also interacts with the HisQ-HisM-HisP complex (4). This provides supportive evidence that this region of HisJ and LAO has maintained homology because of its necessity to interact with the same set of membrane-bound proteins. Then, mutations suppressing the defect in *hisJ5625* should be useful in determining the sites of membrane-receptor contact; identifying the nature of these mutations will help define the protein domains involved in this interaction. All of the suppressor mutations isolated to date are located in the *hisP* gene. This result is particularly interesting since the HisP protein belongs to a family of homologous ATP-binding proteins present in each periplasmic permease and probably involved in energy coupling (1, 6). Since they are thought to be peripheral and possibly located exclusively on the inner surface of the cytoplasmic membrane (20; Kerppola and Ames, unpublished), it is particularly interesting and puzzling how suppressor mutations in such a protein can arise which correct a defect in the distantly located periplasmic HisJ protein. One possibility is that a portion of HisP is somehow accessible to the periplasmic surface of the membrane; alternatively, the ability of *hisP* mutants to suppress a defect in the periplasmic component may reflect interactions communicated via the other two proteins of the membrane-bound complex. Obviously, the isolation of a large number of mutants located in the domains forming the interaction site of HisJ, and of their suppressors, will be necessary in order to define with precision the molecular mechanism of protein-protein interaction. Elegant genetic studies attacking this problem have been performed also for the maltose operon (20).

A useful application of PCR is the ability to amplify DNA from one organism by using primers homologous to another related organism. We have shown this to be true for the amplification of *E. coli* DNA by using *S. typhimurium* primers. These two species are about 150 million years apart in the evolutionary scale and the divergence of their DNA at silent sites is ~58% (14). It may be possible to amplify DNA from organisms even further removed than these, perhaps by utilizing longer primers.

In conclusion, we show that PCR can be used to amplify DNA fragments from the intact bacterial genome and that these fragments can be used for sequencing purposes or subcloning strategies. We have developed a modification of the amplification method that allows direct bulk sequencing of the asymmetrically amplified product. The availability of this technology speeds up enormously the analysis of chromosomal mutant sites which can be easily either sequenced or subcloned into appropriate vectors for a variety of purposes, such as overexpression of proteins and regulatory studies. Cloning of genes is greatly simplified, once their sequence (or of neighboring regions) is known; only the organism to be used as a source of DNA is required. The sequence information needed for designing the primers can also be derived from that of any closely related organism.

ACKNOWLEDGMENTS

We thank R. Higuchi, T. Kocher, and U. Gyllensten for many helpful discussions; A. Wilson for use of the DNA Thermal Cycler; and K. Nikaido for constructing plasmid pFA89 and analyzing the protein product.

This research was supported by Public Health Service grants DK12121 to G.F.-L.A. from the National Institutes of Health and CA39910 to B. N. Ames from the National Cancer Institute.

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