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Preliminary Characterization of a Food-Borne Multiple-Antibiotic-Resistant *Salmonella typhimurium* Strain†

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Plasmid characterization studies were conducted on a *Salmonella typhimurium* strain isolated from pasteurized milk and from a symptomatic patient during the 1985 Illinois salmonellosis outbreak. This strain (Hf) was reported to possess an unusual plasmid profile which distinguished it from all *Salmonella* strains isolated in the United States prior to 1984. Antibiotic susceptibility testing revealed that the strain was resistant to tetracycline, erythromycin, clindamycin, sulfisoxazole, sulfadiazene, triple sulfa, cefoperazone, streptomycin, mezlocillin, piperacillin, carbenicillin, penicillin, ampicillin, and kanamycin. Plasmid analysis revealed that the strain possessed four plasmids with sizes of approximately 158, 98, 10.2, and 6.0 kilobase pairs (kb). Successive transfer at 43°C led to increased antibiotic sensitivity in 75.5% of the isolates screened. Electroporation and calcium chloride treatment were each used to transform plasmid-free *Escherichia coli* strains with the plasmid pool from *S. typhimurium* Hf. Plasmids introduced by transformation ranged in size from 4.4 to 23.2 kb and correlated with resistance to penicillin G, ampicillin, carbenicillin, cephalothin, cefoperazone, cefamandole, mezlocillin, piperacillin, and in some cases, tetracycline and kanamycin. DNA-DNA hybridization experiments localized these resistance genes to a highly duplicated 6.3-kb fragment of the total *Eco*RI restriction digest of the *S. typhimurium* Hf plasmid pool.

In March and April 1985, pasteurized milk contaminated with *Salmonella typhimurium* was responsible for what has been described as the largest outbreak of salmonellosis ever recorded in the United States (16). The outbreak triggered an intensive multiagency investigation which eventually traced the epidemic *S. typhimurium* strain (herein referred to as *S. typhimurium* Hf) to two brands of contaminated 2% low-fat milk from a single Illinois dairy processing plant.

A key feature of *S. typhimurium* Hf which enabled investigators to trace clinical *Salmonella* isolates to the contaminated milk products was the plasmid profile of the strain, which distinguished it from all of the Centers for Disease Control and U.S. Department of Agriculture *Salmonella* reference strains previously isolated. Investigators determined that *S. typhimurium* Hf was the causative organism in at least three outbreaks in Illinois over a period of 8 months, resulting in over 168,000 cases of salmonellosis and two deaths. The antimicrobial drug resistance of this strain was also reported to have increased the magnitude of the 1985 Illinois outbreak (16). National studies conducted by the Centers for Disease Control revealed that the proportion of outbreak *Salmonella* strains resistant to one or more antibiotics increased significantly from 16% in a 1979 to 1980 study to 24% in a 1984 to 1985 survey (10).

Since the discovery of drug resistance plasmids in outbreak *Shigella* strains by Ochiai et al. (15) and Akiba et al. (1) in 1960, resistance plasmids have been identified in many gram-positive and gram-negative bacterial pathogens. The existence of enterobacterial gene transfer systems, including conjugation, transformation, and transduction, as well as transposable elements which code for resistance to multiple antibiotics (5), is believed to greatly increase the accessibility of the microbial gene pool, particularly among related species. Concern about the reported increased drug resistance of outbreak *Salmonella* strains in the United States, along with an awareness of the impact which bacterial drug resistance had upon the magnitude of the 1985 milkborne outbreak, prompted this study of the genetic basis of antibiotic resistance in *S. typhimurium* Hf.

MATERIALS AND METHODS

Bacterial strains. The strains used or constructed in this study are described in Table 1. *S. typhimurium* Hf-PI, originally isolated from unopened pasteurized 2% milk processed during the outbreak (product isolate), was obtained from the Food and Drug Administration, Minneapolis, Minn. *S. typhimurium* Hf-CI, originally isolated from a patient with salmonellosis during the outbreak (clinical isolate), was supplied by M. P. Doyle, Food Research Institute, University of Wisconsin, Madison.

The standard bacterial strains used for transformation studies were supplied by A. Das, University of Minnesota, and included: *Escherichia coli* LE392 (*recA*$^+$ [11]), *E. coli* HB101 (*recA* [4]), and *E. coli* DH5α (*recA*), a derivative of DH5 (6). All *S. typhimurium* and *E. coli* strains were propagated at 37°C in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.).

Antibiotic susceptibility testing. The MICs of 21 different antibiotics were determined with UniScept MIC gram-negative test kits (Analytab Products, Plainview, N.Y.). Susceptibilities to six additional antibiotics were determined by in vitro dose response tests in BHI broth supplemented with 1 to 1,000 μg of the appropriate filter-sterilized antibiotic per ml or by plating the cells in the presence of standard Kirby-Bauer disks (2). In order to encourage the maintenance of drug resistance genes, parental *S. typhimurium* Hf strains were propagated in BHI broth or agar supplemented with the following routine screening antibiotics: ampicillin (50 μg/ml), carbenicillin (50 μg/ml), penicillin G (50 μg/ml), streptomycin (50 μg/ml), sulfisoxazole (50 μg/ml), tetracycline (50 μg/ml), and erythromycin (45 μg/ml).

Plasmid DNA isolation. The most satisfactory results for

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TABLE 1. Bacterial strains used or constructed in this study

Strain	Description	Antibiotic resistance pattern[a]	Plasmid profile (kb)	Reference
S. typhimurium Hf-PI	Outbreak product isolate	Ap, Cb, Cz, Em, Kn, Mz, Pc, Pi, Sm, Su, Tc	158, 98, 10.2, 6.0	16
E. coli LE392	*recA*$^+$ recipient	Em, Su	Plasmid-free	11
E. coli LP1	LE392 transformant	Ap, Cb, Cf, Cz, Ma, Mz, Pc, Pi, Tc, Em, Su	10.8, 8.7, 7.5, 5.8, 4.4	This study
E. coli LC7	LE392 transformant	Ap, Cb, Cf, Cz, Ma, Mz, Pc, Pi, Tc, Em, Su	8.7, 7.4, 4.4	This study
E. coli LC7-10	Cured derivative of LC7	Em, Su	Plasmid-free	This study
E. coli DH5α	*recA* recipient	Su	Plasmid-free	6
E. coli D15C	DH5α transformant	Ap, Cb, Cf, Cz, Kn, Ma, Mz, Pc, Pi, Tc, Su	10.8, (7.4)[b], 4.4	This study
E. coli D3P	DH5α transformant	Ap, Cb, Cf, Cz, Ma, Mz, Pc, Pi, Tc, Su	7.4, 4.4	This study
E. coli D3P-6	Cured derivative of D3P	Su	Plasmid-free	This study
E. coli HB101	*recA* recipient	Em, Sm, Su	Plasmid-free	4
E. coli HC3	HB101 transformant	Ap, Cb, Em, Kn, Mz, Pc, Pi, Sm, Su	23.2, 10.2, (6.0)[b]	This study
E. coli HC3-8	Cured derivative of HC3	Em, Sm, Su	(10.2)[b], 6.0	This study

[a] Abbreviations: Ap, ampicillin; Cb, carbenicillin; Cf, cephalothin; Cz, cefoperazone; Em, erythromycin; Kn, kanamycin; Ma, cefamandole; Mz, mezlocillin; Pc, penicillin; Pi, piperacillin; Sm, streptomycin; Su, sulfisoxazole; Tc, tetracycline.
[b] Parentheses indicate a very faint plasmid band.

plasmid extraction were obtained by using the alkaline lysis procedure of Birnboim and Doly (3). Plasmid DNA from large-scale preparations was purified through cesium chloride-ethidium bromide density gradients as described by Maniatis et al. (11). The DNA was concentrated and desalted by several washes with 10 mM Tris–1 mM EDTA buffer (pH 8.0) in Centricon microconcentrators (Amicon Corp., Danvers, Mass.).

All plasmid DNAs were electrophoresed on 0.5% agarose gels (SeaKem ME agarose; FMC Corp., Marine Colloids Div., Rockland, Maine) by using a Bio-Rad Sub Cell horizontal electrophoresis apparatus (Bio-Rad Laboratories, Richmond, Calif.). Electrophoresis was carried out at 60 V (4 V/cm) for 6 h in Tris-acetate buffer (40 mM Tris, 12 mM sodium acetate, 1 mM disodium EDTA [pH 8.0]). Gels were stained with ethidium bromide (0.5 μg/ml) and photographed under UV transillumination with Polaroid type 107 film.

Plasmid curing trials. Cultures were propagated in BHI broth containing sublethal doses of novobiocin (1,400 μg/ml), ethidium bromide (3 μg/ml), acridine orange (1.5 μg/ml), or sodium dodecyl sulfate (8% [wt/vol]) or in unsupplemented BHI broth incubated at elevated growth temperatures (43 to 44°C [20]). After six to nine consecutive 24-h growth cycles, cells were diluted and plated onto BHI agar plates. Two hundred to five hundred isolates were replica plated onto BHI agar and BHI agar containing the seven routine screening antibiotics, incubated at 37°C for 24 h, scored for the loss of drug resistance, and screened for the loss of plasmid DNA.

Transformation. Transformation of *E. coli* LE392 and DH5α by electroporation was performed under the optimized conditions described by McIntyre and Harlander (12) by using a BTX Transfector 100 (Biotechnologies and Experimental Research, Inc., San Diego, Calif.). Recipient cells were subjected to a single 5-ms pulse at a field strength of 14 kV/cm in the presence of the gradient-purified plasmid pool from *S. typhimurium* Hf-PI. After a 1-h recovery period in BHI broth at 37°C, the cells were plated onto BHI agar containing the routine screening antibiotics, either individually or in combination. Plates were incubated for 48 h at 37°C.

Transformation of logarithmic-phase *E. coli* HB101 cells was performed by the calcium chloride treatment procedure of Maniatis et al. (11). Cells were plated onto BHI agar containing penicillin (50 μg/ml), carbenicillin (50 μg/ml), tetracycline (50 μg/ml), or kanamycin (45 μg/ml) or the four drugs in combination, and were incubated for 48 h at 37°C.

After transformation by electroporation or calcium chloride treatment, representative colonies from each selective agar plate were transferred to BHI broth containing the appropriate antibiotic and were incubated at 37°C for 18 to 24 h. Cells from turbid broths were restreaked for isolation on BHI agar containing the appropriate antibiotic and were incubated at 37°C overnight. Fifteen to 20 individual colonies were selected and tested for additional drug resistances and for the presence of plasmid DNA.

Selected *E. coli* transformants were cured of plasmids by propagation in BHI broth at 43°C for three to six consecutive growth cycles.

Plasmid analysis. Selected plasmids were digested with the restriction endonuclease *Eco*RI (Promega Biotec, Madison, Wis.). Restriction fragments were separated by electrophoresis on 0.6% agarose gels run at 60 V (4 V/cm) for 6 h.

Southern transfer of intact plasmids and restriction fragments to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) and hybridization reactions were carried out as described by Maniatis et al. (11). Gradient-purified [^{32}P]dCTP-labeled plasmid probes were prepared by the random primer method by using the Prime-a-Gene labeling system (Promega) according to the specifications of the manufacturer.

RESULTS

Antibiotic susceptibility testing. The Centers for Disease Control and the Food and Drug Administration had previously demonstrated resistance of *S. typhimurium* Hf to eight antimicrobial drugs (16). In this study, resistance to six additional antibiotics was demonstrated, including erythromycin (MIC, 8 μg/ml), clindamycin (MIC, >8 μg/ml), piperacillin (MIC, >512 μg/ml), cefoperazone (MIC, >64 μg/ml), mezlocillin (MIC, >256 μg/ml), and kanamycin (MIC, 50 μg/ml).

The drug resistance of *S. typhimurium* Hf was further characterized by challenging the organism with routine screening antibiotic concentrations which were 10 to 6,000 times higher than those included in the MIC gram-negative test kits. Dose-response tests revealed that the strain was

capable of growth in the presence of antibiotic concentrations which were 20 to 6,000 times higher than the concentrations which a strain must tolerate in order to be designated resistant by the National Committee for Clinical Laboratory Standards (13; data not shown).

Plasmid profile analysis. Plasmid profile analyses of the product and clinical isolates revealed that both isolates possessed four plasmids with molecular sizes of approximately 158, 98, 10.2, and 6.0 kilobase pairs (kb) (data not shown). Previous investigators reported a plasmid profile of 211, 98, and 3.0 kb for the outbreak strain, although two strains possessed an additional 68-kb plasmid and at least one isolate was missing the 3.0-kb plasmid (16). The 10.2-kb plasmid identified during this study was consistently present in the plasmid profiles of both outbreak isolates. Moreover, the 10.2-kb plasmid band remained after purification of the DNA by ethidium bromide-cesium chloride density gradient centrifugation and after treatment with exonuclease III (650 U of exonuclease III, 37°C for 4 h; data not shown), a DNase which degrades circular and linear forms of DNA. The difference in reported sizes may be due, in part, to differences in gel electrophoresis conditions and molecular weight standards used.

Plasmid curing trials. To obtain phenotypic evidence of linkage of antibiotic resistance genes to plasmid DNA, attempts were made to cure *S. typhimurium* plasmids. Hf-PI was not amenable to curing by novobiocin, intercalating dyes, sodium dodecyl sulfate, or propagation in antibiotic-free media under the conditions used. Propagation at elevated growth temperature was the only effective curing method used in this study. After nine consecutive 24-h growth cycles at 44°C, 228 of 302 *S. typhimurium* Hf-PI isolates (75.5%) failed to grow on BHI agar containing the routine screening antibiotics. The plasmid profiles of 68 of the presumptive cured isolates were determined. In each case, sensitivity to the routine screening antibiotics in combination was accompanied by the apparent loss or deletion of the 98-kb parental plasmid (data not shown). MIC testing of 20 of these isolates revealed increased sensitivity to erythromycin (the MIC of erythromycin decreased from 85 μg/ml to 45 μg/ml). Resistance to penicillin, semisynthetic beta-lactams, and other cephalosporins remained unchanged. Therefore, the presence of the 98-kb plasmid appeared to be necessary for growth on BHI agar containing erythromycin at concentrations of ≥45 μg/ml. Attempts to isolate a derivative which had been cured of the 10.2- or 6.0-kb plasmids were unsuccessful.

Transformation. In order to investigate the linkage of other antibiotic resistance determinants to plasmid DNA, attempts were made to independently transfer the plasmids from *S. typhimurium* Hf to alternative hosts. The transformation of three different drug-susceptible, plasmid-free *E. coli* strains was conducted. The frequencies of transformation were comparable when *recA*$^+$ (*E. coli* LE392) or *recA* (*E. coli* DH5α) were used. Thus, the maintenance and expression of the resistance determinants did not appear to be *recA* dependent. The frequencies of transformation of the recipients ranged from 3×10^{-5} to 5×10^{-6} transformants per input cell. Resistance to penicillin G, semisynthetic penicillins, cephalosporins of the first generation (i.e., cephalothin), second generation (i.e., cefamandole), and third generation (i.e., cefoperazone), and tetracycline was transferred en bloc by electroporation to both recipients. The *E. coli* DH5α transformants typified by strain D15C (Table 1) also acquired resistance to kanamycin.

In all cases, transformation to multiple antibiotic resistance was accompanied by the acquisition of plasmid molecules ranging from 4.4 to 10.8 kb in size. These plasmids did not correspond in size to any of the four input *S. typhimurium* Hf plasmids, indicating that the donor plasmids may be present in deleted or cointegrated forms in the *E. coli* hosts (Table 1). When selected transformants were cured of plasmid DNA, the original susceptible phenotypes were restored (Table 1).

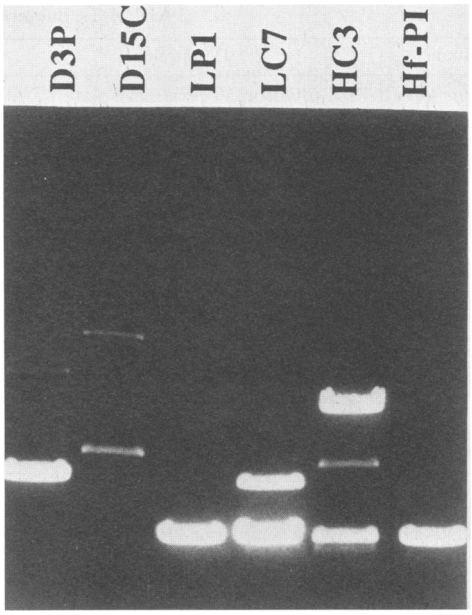

FIG. 1. Gel electrophoresis of *Eco*RI fragments of plasmids isolated from representative *E. coli* transformants and *S. typhimurium* Hf-PI. *E. coli* transformants: D3P, D15C, LP1, LC7, and HC3. *S. typhimurium*, Hf-PI. Lambda DNA digested with *Hin*dIII served as molecular weight standards.

The calcium chloride-induced transformation of *E. coli* HB101 cells resulted in the appearance of a single class of drug-resistant transformants. These transformants, typified by strain HC3, possessed plasmids of 23.2 and 10.2 kb, plus a very faint band at the 6.0-kb position, and were resistant to penicillin G, semisynthetic beta-lactams, and kanamycin. Curing by incubation at an elevated growth temperature resulted in drug-susceptible isolates (typified by strain HC3-8) which had lost the 23.2-kb plasmid. A faint band of 10.2 kb and a much more distinct band of 6.0 kb appeared in the profile of strain HC3-8 (data not shown).

Analysis of *E. coli* transformants. Purified plasmid DNA from each of the five representative *E. coli* transformants was digested with *Eco*RI and subjected to agarose gel electrophoresis (Fig. 1). Digestion of the plasmids from *E. coli* D3P generated a single 4.4-kb fragment, which was labeled for use as a drug resistance gene probe in all subsequent hybridization experiments. This fragment was considered suitable for use as a probe because of its relatively small size, its purity, and its association with the resistance of *E. coli* D3P to nine antibiotics (Table 1).

The ^{32}P-labeled probe hybridized to the 4.4-kb fragment common to the *Eco*RI digests of the plasmids from *E. coli* transformants LP1, LC7, and D15C. The probe also hybridized to the 10.5-kb fragment from *E. coli* D15C and to the 15.5-, 10.5-, and 7.5-kb fragments from *E. coli* HC3. The hybridization data indicated that the 4.4-kb probe sequence (or a portion thereof) was maintained on multiple *Eco*RI

TABLE 2. Fragments resulting from *Eco*RI digestion of plasmids from *S. typhimurium* Hf-PI and selected *E. coli* transformants

Source of plasmid DNA	Detectable fragments (kb)[a]
S. typhimurium Hf-PI (undigested plasmids)	158, 98, 10.2*, 6.0*
S. typhimurium Hf-PI	14.5, 13.0, 12.5, 10.5, 9.6, 8.5, 6.3*, 5.3, 3.3
E. coli LP1	6.1, 4.4*
E. coli LC7	4.4*
E. coli D15C	10.5*, 7.2, 4.4*
E. coli D3P	4.4*
E. coli HC3	15.5*, 10.5*, 7.5*

[a] Unless otherwise specified, fragments listed are those generated after digestion of gradient-purified total plasmid DNA from each strain. *, A fragment which hybridized to the 4.4-kb radiolabeled probe from *E. coli* D3P.

fragments of the plasmids from strains D15C and HC3 (Table 2).

By using the same 4.4-kb probe, a Southern hybridization experiment was conducted against undigested total DNA from *S. typhimurium* Hf-PI and against *Eco*RI-digested total plasmid DNA from *S. typhimurium* Hf-PI. Autoradiographs revealed that the probe hybridized strongly to both the 10.2- and 6.0-kb intact plasmids from *S. typhimurium* Hf-PI (data not shown). *Eco*RI digestion of total plasmid DNA from *S. typhimurium* Hf-PI yielded nine detectable fragments (Fig. 1). Of these fragments, only the highly duplicated 6.3-kb fragment hybridized to the 4.4-kb probe (Table 2). These two observations indicate that the 10.2- and 6.0-kb plasmids from *S. typhimurium* Hf-PI possess a common determinant which encodes resistance to ampicillin, carbenicillin, cefoperazone, mezlocillin, penicillin G, piperacillin, and tetracycline. The antibiotic resistance determinants expressed in the *E. coli* transformants appeared to have originated only from the 6.0- and 10.2-kb plasmids from *S. typhimurium* Hf-PI.

DISCUSSION

The appearance in 1985 of an epidemic *S. typhimurium* strain with a unique plasmid profile presented an opportunity to investigate the importance of extrachromosomal DNA in mediating *Salmonella* drug resistance. In this study, transformation and DNA-DNA hybridization techniques were used to demonstrate that the resistance of *S. typhimurium* Hf to ampicillin, carbenicillin, cefoperazone, kanamycin, mezlocillin, piperacillin, and tetracycline was mediated by determinants present on both the 10.2- and 6.0-kb plasmids. These two plasmids were not amenable to curing by the methods used in this study. Curing experiments did, however, correlate the 98-kb plasmid with resistance to erythromycin at concentrations of $\geq$45 μg/ml.

The transformation of three different drug-susceptible *E. coli* strains with the plasmid pool from *S. typhimurium* Hf resulted in the appearance of transformants that had acquired resistance to penicillin G, ampicillin, carbenicillin, cephalothin, cefoperazone, cefamandole, mezlocillin, piperacillin, and in some cases, kanamycin and tetracycline. When screened for the presence of plasmid DNA, the *E. coli* transformants were found to possess a variety of plasmids, which ranged in size from 4.4 to 23.2 kb. The *S. typhimurium* Hf plasmids appeared to be maintained in deleted or cointegrated forms in the *E. coli* hosts. Plasmid curing by propagation at elevated growth temperature provided an effective means of confirming the role of extrachromosomal DNA in mediating the multiple drug resistances expressed in the *E. coli* transformants (Table 1).

DNA-DNA hybridization experiments revealed that the 4.4-kb probe shared sequence homology with all of the 4.4-kb *Eco*RI fragments present in the other DH5α and LE392 transformants (Table 2). The probe also hybridized to the undigested 10.2- and 6.0-kb plasmids from *S. typhimurium* Hf, indicating that these two *Salmonella* plasmids possessed common antibiotic resistance determinants.

The mechanism(s) of antibiotic resistance coded for by the 4.4-kb fragment is not known at this time. Of the nine antibiotics for which *E. coli* transformants are resistant, all except tetracycline belong to the broad class of beta-lactam antibiotics. The production of beta-lactamases is the most common mechanism of resistance (14). In this study, the parental strain, as well as the *E. coli* transformants, were resistant to cefamandole and cefoperazone beta-lactam antibiotics. To date, only 1 of the 20 different plasmid-mediated beta-lactamases which have been studied confers resistance to these broad-spectrum cephalosporins (8, 17, 18, 21). In addition, it is not currently known whether or not the antibiotic resistance genes are located on a transposon. Transposable elements as small as 2.5 kb in size (Tn9) have been isolated from strains of *E. coli* (9). To date, attempts to transfer the antibiotic-resistant phenotype from *S. typhimurium* Hf to an F^- Cm^r *E. coli* recipient by conjugation in either broth or on filters have been unsuccessful (data not shown). Further characterization of the mechanism(s) of resistance and the linkage of the antibiotic resistance gene(s) to transposons is currently under investigation in our laboratory.

The acquisition of antibiotic resistance genes which reside on plasmids or transposons is considered an important genetic survival mechanism for microorganisms subjected to environments of intense antibiotic-selective pressure. Because antibiotics are frequently added to animal feeds to treat and prevent infection and to promote growth (19), it is feasible that multiple-antibiotic-resistant strains such as *S. typhimurium* Hf could emerge from livestock farms and enter the food supply via foods of animal origin. Holmberg and co-workers (7) have identified food animals as the source of 69% of resistant and 46% of sensitive *Salmonella* strains responsible for salmonellosis outbreaks in the United States with identifiable sources. Over one-half of the outbreaks caused by the transmission of salmonellae from animals to humans were caused by drug-resistant strains. The expression of multiple antibiotic resistance in bacterial pathogens responsible for major epidemics represents a growing obstacle to the treatment of human and animal infections. This study reaffirms the importance of plasmids in mediating the broad-spectrum beta-lactam, kanamycin, tetracycline, and erythromycin resistance of a *Salmonella* strain responsible for a food-borne outbreak of unprecedented public health impact.

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