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Bacillus subtilis Bacteriophages SP82, SPO1, and φe: a Comparison of DNAs and of Peptides Synthesized During Infection

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The genomes of *Bacillus subtilis* phages φe, SPO1, and SP82 were compared by DNA-DNA hybridization, analysis of DNA fragments produced by digestion with restriction endonucleases, comparison of the arrays of peptides synthesized during infection, and phage neutralization. DNA-DNA hybridization experiments indicated that about 78% of the SP82 DNA was homologous with SPO1 DNA, whereas 40% of the φe DNA was homologous to either SPO1 or SP82 DNA. Agarose gel electrophoresis was used to compare the molecular weights of DNA fragments produced by cleavage of SP82, SPO1, and φe DNAs with the restriction endonucleases *Hae* III, *Sal* I, *Hpa* II, and *Hha* I. Digestion of the DNAs with *Hae* III and *Sal* I produced only a few fragments, whereas digestion with *Hpa* II and *Hha* I yielded 29 to 40 fragments, depending on the DNA and the enzyme. Comparing the *Hpa* II fragments, 51% of the SP82 fragments had mobilities which matched those of SPO1 fragments, 32% of the SP82 fragments matched the φe fragments, and 34% of the SPO1 fragments matched the φe fragments. Comparing the *Hha* I digestion products, 62% of the SP82 fragments had mobilities matching the SPO1 fragments, 24% of the SP82 fragments matched the φe fragments, and 22% of the SPO1 fragments matched the φe fragments. Analysis of peptides by electrophoresis on one-dimensional sodium dodecyl sulfate-polyacrylamide slab gels showed that approximately 70 phage-specific peptides were synthesized in the first 24 min of each infection. With mobility and the intervals of synthesis as criteria, 66% of the different SP82 peptides matched the SPO1 peptides, 34% of the SP82 peptides matched the φe peptides, and 37% of the SPO1 peptides matched the φe peptides. Phage neutralization assays using antiserum to SP82 yielded *K* values of 510 for SP82, 240 for SPO1, and 120 for φe.

SP82, SPO1, and φe are members of a group of large, hydroxymethyluracil-containing, virulent bacteriophages that infect *Bacillus subtilis* (11). Earlier studies demonstrated that the phages of this group cannot be distinguished on the basis of morphology, neutralization (2), or the physical properties of phage DNA (sedimentation coefficients, melting points, guanine-plus-cytosine content, and buoyant densities [25]). Slight differences were found in plaque morphology (25) and in the efficiency of adsorption or plating on certain mutant strains of *B. subtilis* (27). The current genetic maps for these phages are not identical (11), although only about one-third of the total probable SP82 and SPO1 cistrons have been identified. The extent of hybridization of SPO1 DNA to SP82 DNA has not been reported. However, hybridization experiments using the DNA of phage 2C, another member of this group, demonstrated that SPO1 and SP82 were 100% homologous with 2C, whereas φe DNA was 83% homologous with 2C (25).

Infections with SP82 and SPO1 have been of particular interest with regard to the mechanisms that regulate transcription. Analyses by competition hybridization revealed the synthesis of similar temporal RNA classes in cultures infected with SPO1 (9) or SP82 (23). Modification of the subunit composition of the *B. subtilis* RNA polymerase has been shown to be a major determinant of the specificity of transcription in both infections (5-8, 17, 22, 23, 26). The polymerases isolated from SP82- and SPO1-infected cells appear to have the same subunit structure and similar specificities of in vitro transcription (12, 17).

In view of the increasing number of investigations with SPO1 and SP82, additional studies to determine the extent of the relatedness of

these phages would be of value. The present communication reports investigations which compare SPO1 and SP82 and include φe, a more distantly related phage of the same group. The extent of relatedness of these three phages has been examined by means of DNA-DNA hybridization, by comparing the fragments produced by digestion of phage DNAs with four restriction endonucleases, by comparing the patterns of peptides synthesized during infection by each phage, and by determining the rates of neutralization by SP82 antiserum.

MATERIALS AND METHODS

Phage growth. The methods used for the growth and lysis of *Bacillus subtilis* 168 try⁻ have been described (13). The time of lysis in the defined medium used for protein labeling was 45 min for SP82, 50 min for SPO1, and 85 min for φe. All three phages were obtained from Charles R. Stewart.

Phage neutralization. The preparation of SP82 antiserum (19) and the determination of rate constants for phage neutralization (1) have been described.

Digestion of phage DNA with restriction endonucleases. The procedures employed for purification of phages and phage DNA, digestion of phage DNA with restriction endonucleases *Hae* III, *Sal* I, and *Hpa* II, agarose gel electrophoresis of DNA fragments, ethidium bromide staining, and photography of gels have been described (16). To digest DNAs with *Hha* I (New England Biolabs, Beverly, Mass.), 1.5 μg of phage DNA was incubated for 10 h at 37°C with 3 U of *Hha* I in 50 mM NaCl, 6 mM Tris (pH 7.5), 6 mM $MgCl_2$, 6 mM β-mercaptoethanol, and 10 μg of bovine serum albumin in a total volume of 100 μl.

Peptide analysis. Cultures (50 ml each) were grown to a density of approximately 2×10^8 cells per ml, irradiated with UV (13 ergs/mm² per s for 4 min with gentle agitation), and incubated at 37°C for 5 min before phage infection at a multiplicity of about 20. This treatment was sufficient to decrease the synthesis of host proteins by approximately 75% without changing the time of lysis. Labeling of phage-infected cells with [^{35}S]methionine, the preparation of samples for electrophoresis on one-dimensional sodium dodecyl sulfate-polyacrylamide slab gels, and autoradiography have been described (12). The identity of peptide bands on autoradiograms was determined by lining up peptides across the autoradiograms and, in some instances, by superimposing autoradiograms.

Two-dimensional electrophoresis was performed by the method of O'Farrell (18). Samples labeled with [^{35}S]methionine were prepared as described for one-dimensional electrophoresis and diluted into the O'Farrell lysis buffer before isoelectric focusing. Electrophoresis after focusing was performed on 9-cm sodium dodecyl sulfate-polyacrylamide slab gels by using a linear gradient of 7 to 15% acrylamide in 0.8% bisacrylamide; the procedure for autoradiography was as described earlier (12). The identity of peptide spots on the autoradiograms was determined by superimposing the autoradiograms obtained from SPO1- and SP82-infected cells, matching the spots, and comparing them with an autoradiogram of mixed SPO1- and SP82-labeled peptides.

Isolation of $^{32}PO_4$-labeled SP82 DNA. A 5-ml culture of SP82-infected *B. subtilis* grown in a phosphate-depleted medium (14) was labeled by the addition of 20 μCi of $^{32}PO_4$ (carrier-free; New England Nuclear Corp.) per ml. Growth was stopped 25 min later by the addition of ice and NaN_3 (final concentration, 0.01 M), and the cells were collected by centrifugation. DNA was extracted as described earlier (14), except that 1.25 mg of calf thymus DNA was added as a carrier, and the preparation was digested with RNAse (15 μg of RNAse A per ml and 15 U of RNAse T1 per ml, incubated at 37°C for 30 min) after the initial extraction.

DNA-DNA hybridization. The method of Crosa et al. (3) was used with the following modification: approximately 0.1 μg of sheared, denatured, $^{32}PO_4$-labeled DNA was incubated at 65 or 70°C, as indicated, with 150 μg of sheared, denatured, unlabeled DNA in 1 ml of 0.33 M NaCl–0.01 M Tris (pH 7.2) for 20 h. Shearing was performed by sonic oscillation at maximum power for 3 min with the small probe of a Branson model W140 sonicator and yielded DNA fragments of 9 to 16S. The hybridization mixture was cooled, and four 0.2-ml portions were withdrawn and transferred to tubes containing 0.8 ml of buffer (0.38 M sodium acetate [pH 4.5], 0.125 mM $ZnSO_4$, 0.188 M NaCl, and 25 μg of sonically treated, denatured calf thymus DNA per ml). A total of 1,000 U of S1 nuclease (Miles Laboratories, Elkhart, Ind.) was added to each of two samples; the other two samples were used to determine the amount of input radioactivity. The samples were incubated at 50°C for 20 min, precipitated with 1 ml of 10% trichloroacetic acid, 200 μg of yeast RNA was added as carrier, and the precipitates were collected on glass fiber filters. The filters were washed with 30 ml of a solution of cold 5% trichloroacetic acid containing 0.01 M $Na_4P_2O_7$, 0.004 M Na_2PO_4, and 100 μg of ATP per ml and dried, and the radioactivity was determined in a scintillation counter. Control experiments showed that the conditions used for digestion with S1 nuclease resulted in the degradation of 99% of single-stranded DNA and less than 5% of double-stranded DNA.

RESULTS

DNA-DNA hybridization. Digestion with S1 nuclease was selected as the method of measuring the formation of DNA-DNA hybrids to minimize error introduced by nonhybridized regions of labeled, single-stranded DNA attached to hybridized duplexes. As shown by several investigators, for example, by Shenk et al. (21), digestion with S1 nuclease removes single-strand segments bounded by or attached terminally to heteroduplex structures. Hybridizations were performed under conditions of excess unlabeled DNA, which yielded approximately 80% hybridization of added radioactivity in the homologous reaction.

The reciprocal heterologous hybridizations (i.e., labeled SP82 DNA versus unlabeled SPO1

DNA, compared with labeled SPO1 DNA versus unlabeled SP82 DNA) showed a variation of approximately 10% (Table 1). The reason for this variability is not known but, as discussed below, it was not due to differences in the sizes of the phage genomes. If the values for the reciprocal hybridizations are averaged, it is seen that at 65°C, 84% of the SPO1 and SP82 DNAs hybridize, 50% of the φe DNA anneals with SPO1 DNA, and 47% of the φe DNA hybridizes with SP82 DNA. Hybridization reactions were also incubated under more stringent conditions (70°C) to reduce the amount of mismatching. At this temperature, the amount of input radioactivity binding in homologous hybridizations was reduced by approximately 10%. The average hybridization in heterologous reactions was approximately 78% for SPO1 and SP82, approximately 41% for SPO1 and φe, and approximately 38% for φe and SP82. Reactions were not incubated at temperatures above 70°C because of the marked decrease in hybridization of homologous reactions (e.g., only 45% of the SP82-labeled DNA annealed to unlabeled SP82 DNA at 75°C).

DNA fragments produced by digestion with restriction endonucleases. Digestion of related DNAs with these enzymes detects changes in single base pairs, provided that the changes occur at sequences recognized by the enzymes or occur at other sites to generate new recognition sequences. Figure 1 is a photograph of an agarose gel stained with ethidium bromide, showing the fragments of DNA resulting from the digestion of SP82, SPO1, and φe DNAs with restriction endonucleases *Sal* I and *Hae* III. Before digestion, the DNAs had approximately the same molecular weights (Fig. 1, lanes g, h, and i). However, these DNAs were fragmented during purification and probably represent half-molecules of about 4×10^7 to 5×10^7 molecular weight. The enzyme *Sal* I cleaved at the sequence GTCGAC (the arrow shows the point of cleavage) and produced four fragments from SP82 (Fig. 1, lane a), five fragments from SPO1 (Fig. 1, lane b), and two from φe (Fig. 1, lane c). Digestion with the enzyme *Hae* III, which recognizes the sequence GGCC, produced five fragments from SP82 (Fig. 1, lane d) and six fragments plus two partially digested fragments from SPO1 (Fig. 1, lane e). Six bands were produced from φe (Fig. 1, lane f); when the photographic negative was scanned, however, it was apparent that band C was a doublet.

The sizes of fragments of SP82 produced by *Sal* I and *Hae* III were determined previously, and the fragments were ordered on a physical map of the SP82 genome (15). The molecular weights of the φe and SPO1 fragments (Table 2) were estimated from their mobilities relative to the mobilities of the SP82 fragments. It should be noted that the molecular weights of the large fragments cannot be determined accurately because these fragments migrate in the nonlinear portion of a calibration curve derived by plotting the mobilities of known markers as a function of molecular weight (15).

The restriction endonuclease patterns shown in Fig. 1 demonstrate that the DNAs of SP82, SPO1, and φe contain different sequences, although the molecular weights of some of the fragments correspond. For example, the *Hae* A and B fragments of SP82 are the same size as the *Hae* A and B fragments of SPO1 DNA. Similarly, the *Sal* B fragments of SP82 and SPO1 are the same size, and the migration of *Sal* D of SP82 matches that of *Sal* E produced from SPO1. There is less apparent correlation between the φe fragments and the SP82 or SPO1 fragments. Overall, these comparisons are not particularly useful because *Hae* III and *Sal* I produced few fragments and, as noted above, it is difficult to determine accurately the molecular weights of the larger fragments.

Digestion of SP82, SPO1, and φe DNAs with restriction endonuclease *Hpa* II or *Hha* I produced a large number of fragments (Fig. 2). Digestion with *Hpa* II, which cleaves at CCGG, yielded 37 fragments from SP82 DNA, 29 fragments from SPO1 DNA, and 40 fragments from

TABLE 1. *Hybridization of SP82, SPO1, and φe DNAs*

Source of [^{32}P]-DNA	% Hybridization with following source of unlabeled DNA[a]:						% Input radioactivity annealing in homologous hybridizations at:	
	SP82		SPO1		φe			
	65°C	70°C	65°C	70°C	65°C	70°C	65°C	70°C
SP82	100	100	80	76	50	40	80	66
SPO1	88	80	100	100	46	35	79	70
φe	44	35	53	47	100	100	81	68

[a] Hybridization reaction mixtures described in the text contained the following amounts of input radioactivity: 20,064 cpm of SP82 DNA, 18,039 cpm of SPO1 DNA, and 19,472 cpm of φe DNA. Data are expressed as percent relative to homologous hybridization.

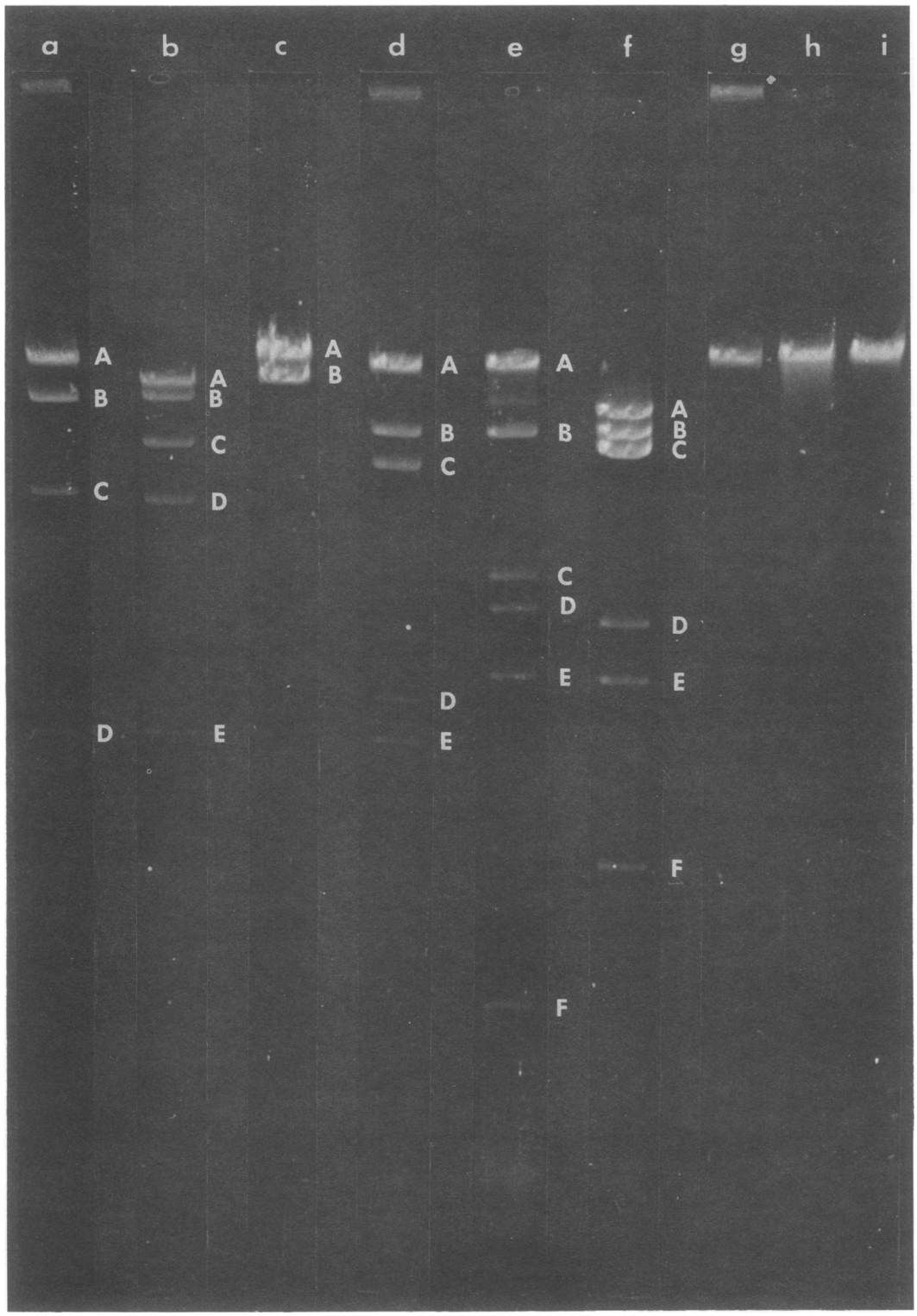

FIG. 1. *Agarose gel analysis of DNA fragments produced by digestion of 0.6 μg of SP82 DNA, 1.0 μg of SPO1 DNA, or 0.8 μg of φe DNA with Sal I or Hae III. Photograph of an ethidium bromide-stained 0.35% agarose slab gel, with the fragments lettered from top to bottom. DNA fragments produced by digestion with Sal I: (a) SP82 DNA, (b) SPO1 DNA, and (c) φe DNA. DNA fragments produced by digestion with Hae II: (d) SP82 DNA, (e) SPO1 DNA, and (f) φe DNA. Undigested controls shown in the last three lanes contained (g) 0.3 μg of SP82 DNA, (h) 0.7 μg of SPO1 DNA, and (i) 0.4 μg of φe DNA.*

TABLE 2. *Molecular weights of fragments produced by digestion of SP82, SPO1, and φe DNAs with Hae III and Sal I*

Fragment	Mol wt (×10⁶)					
	Hae III			*Sal* I		
	SP82	SPO1	φe	SP82	SPO1	φe
A	46	46	23.2	46	32	48
B	19.9	19.9	19.9	27.7	27.7	32
C	14.7	7.65	15.2[a]	11.2	15.2	
D	4.78	6.6	6.3	4.1	10.6	
E	4.13	5.1	5.1		4.1	
F		1.6	2.65			

[a] Fragment C produced during digestion of φe DNA was a doublet.

φe DNA. *Hha* I cleaves at the sequence GCGC and yielded 37 fragments from SP82 DNA, 37 fragments from SPO1 DNA, and 33 fragments from φe DNA. It is obvious that each DNA yielded a distinctive restriction pattern, thereby providing an easy, convenient method of distinguishing SP82, SPO1, and φe.

The molecular weights of the SPO1 and φe DNA fragments and the SP82 fragments produced by digestion with *Hha* I were estimated by comparison with the mobilities of the SP82 fragments resulting from digestion with *Hpa* II (data not shown). The molecular weights of the latter were determined previously (15). Many of the bands contain molecules of identical molecular weight which arise from different regions of the physical map. The possible explanations for the presence of these bands have been discussed previously (15; J. M. Cregg and C. R. Stewart, Virology, in press). The number of copies in such bands was crudely estimated by scanning the photographic negative with a Joyce-Loebl densitometer, determining the area under the peak corresponding to each band, and dividing by the molecular weight. Summation of the molecular weights of the *Hpa* II fragments, including the multiple copies, yielded a molecular weight of 9×10^7 for SP82, 9.3×10^7 for SPO1, and 9.3×10^7 for φe. Very similar data were obtained by summation of the molecular weights of the *Hha* I fragments. The molecular weight for SP82 DNA agrees with estimates of genome size determined by electron microscopy (10). The value for SPO1 agrees with the molecular weight determined by summation of *Eco*RI* cleavage products (Cregg and Stewart, Virology, in press); a slightly higher value was reported by Talkington and Pero (24).

Tables 3 and 4 compare the pairs of phage DNAs by listing the restriction fragments having the same molecular weights (the fragments are identified by the band numbers shown in Fig. 2). Of the 37 SP82 fragments, 19 (51% of the total number of fragments) produced by *Hpa* II can be matched by mobilities with SPO1 fragments produced by digestion with this enzyme. Summation of the molecular weights of the matched SP82 and SPO1 fragments, including multiple copies, yielded a rough estimate of 4.4×10^7, or approximately 48% of the total genome. Of the 29 SPO1 fragments 10 (or 34% of the total number of fragments) produced by digestion with *Hpa* II matched the mobilities of φe fragments generated by digestion with *Hpa* II (Table 4); the summated molecular weights of these matched fragments were approximately 1.8×10^7 for SPO1, or approximately 19% of the genome. Similarly, 12 of the 37 SP82 fragments (or 32% of the total number) matched the mobilities of φe DNA fragments; the summated molecular weight of the matched SP82 fragments was 1.7×10^7 or approximately 19% of the SP82 genome. A parallel comparison of the *Hha* I bands (Table 4) shows that 23 of the 37 SP82 bands matched the SPO1 bands (62% of the total number; 64% of the molecular weight of the genome); 8 of the 37 SPO1 fragments matched the φe fragments (22% of the total number of fragments; 8% of the total molecular weight of the SPO1 genome); and 9 of the SP82 fragments matched the φe DNA fragments (24% of the total number and 18% of the total molecular weight of the SP82 genome).

The experiments described above on the extent of sequence homology and analysis by digestion with restriction endonucleases do not provide information on the effect of differences in base sequences on gene products. This question was examined by paired comparisons of the peptides synthesized during infection by using electrophoresis on one-dimensional sodium dodecyl sulfate-polyacrylamide slab gels and by comparing the rates of phage neutralization.

Analysis of peptides synthesized during phage infection. Earlier studies on the synthesis of peptides in SPO1-infected cultures (16) and more recent investigations of the infection with SP82 (12) demonstrated that many proteins are synthesized and that the patterns of gene expression are complex.

Figures 3 to 5 present direct comparisons of peptides synthesized during infection of *B. subtilis* with SP82, SPO1, and φe. A total of approximately 70 phage-specified peptides could be detected in each infection under our conditions of labeling, electrophoresis, and autoradiography during the first 24 min of infection. This accounts for approximately 70% of the estimated coding capacity of these phages. It should be noted that few or no additional peptides were synthesized after 24 min of infection with SP82 (W. R. Hiatt, Ph.D. thesis, University of Washington, Seattle, 1977) and SPO1 (Downard, unpublished data), but this point was not investigated for φe. Additional peptides could be pro-

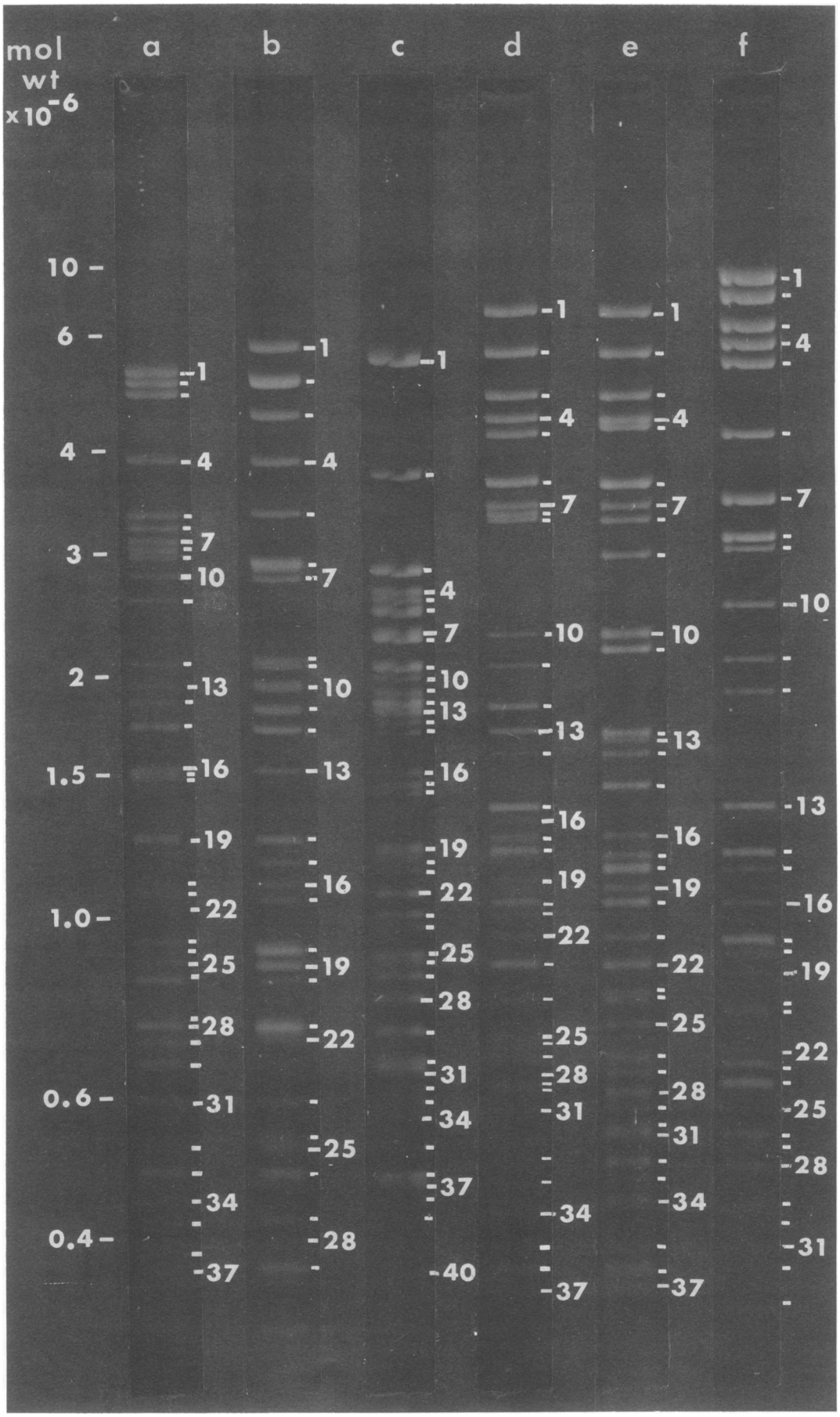
mol wt x10-6
a
b
c
d
e
f
10 –
6 –
4 –
3 –
2 –
1.5 –
1.0 –
0.6 –
0.4 –

TABLE 3. *Molecular weights of matching fragments produced by digestion of SP82, SPO1, and φe DNAs with Hpa II*

SP82	Copies	SPO1	Copies	Mol wt ($\times 10^6$)	SP82	Copies	φe	Copies	Mol wt ($\times 10^6$)	SPO1	Copies	φe	Copies	Mol wt ($\times 10^6$)
2	1	2	2	5.1	12	1	9	2	2.07	6	2	3	2	2.72
4	1	4	1	3.85	15	2	15	1	1.72	9	1	9	2	2.07
5	1	5	1	3.18	16	1	16	1	1.53	12	2	15	1	1.72
10	1	7	1	2.67	21	1	22	3	1.08	13	1	16	1	1.53
12	1	9	1	2.07	25	2	26	2	0.9	19	5	26	2	0.90
13	1	10	2	1.93	28	5	29	3	0.74	21	6	29	3	0.74
15	2	12	2	1.72	30	4	30	4	0.65	23	2	33	2	0.59
16	1	13	1	1.53	31	4	33	2	0.59	25	2	35	2	0.51
19	3	14	3	1.23	32	3	35	2	0.51	26	6	36	1	0.47
20	1	16	2	1.11	33	8	36	1	0.47	27	2	39	1	0.42
24	1	18	6	0.92	34	3	38	2	0.45					
25	2	19	5	0.90	35	1	39	1	0.42					
26	4	20	2	0.84										
28	5	21	6	0.74										
31	4	23	2	0.59										
32	3	25	2	0.51										
33	8	26	6	0.47										
35	1	27	2	0.42										
37	6	29	6	0.36										

TABLE 4. *Molecular weights of matching fragments produced by digestion of SP82, SPO1, and φe DNAs with Hha I*

SP82	Copies	SPO1	Copies	Mol wt ($\times 10^6$)	SP82	Copies	φe	Copies	Mol wt ($\times 10^6$)	SPO1	Copies	φe	Copies	Mol wt ($\times 10^6$)
1	1	1	1	7.2	5	1	6	1	4.18	18	4	15	1	1.15
2	1	2	1	5.8	15	3	13	2	1.37	20	2	16	1	1.05
3	1	3	1	4.78	18	3	14	2	1.19	21	1	17	4	0.95
4	1	4	1	4.4	20	2	16	1	1.05	29	1	25	1	0.57
6	2	6	2	3.43	22	2	17	4	0.95	31	3	26	3	0.54
7	1	7	1	3.29	29	2	24	6	0.62	34	2	29	2	0.45
9	1	8	1	3.15	31	1	25	1	0.57	35	1	31	1	0.39
10	1	10	2	2.25	35	1	31	1	0.39	36	4	32	1	0.36
13	2	12	2	1.69	36	5	32	1	0.36					
14	1	14	1	1.59										
20	2	20	2	1.05										
22	2	21	1	0.95										
23	4	22	3	0.89										
24	2	24	2	0.79										
27	2	26	2	0.67										
28	1	27	1	0.64										
30	2	28	3	0.60										
31	1	29	1	0.57										
32	2	32	3	0.50										
33	1	33	1	0.47										
35	1	35	1	0.39										
36	5	36	4	0.36										
37	2	37	2	0.33										

duced at later times in the latter infection since, under our conditions of growth and infection, this phage had a longer lytic cycle than the other two. A phage-specific peptide was defined as a radioactive band which appeared after addition of phage and had an electrophoretic mobility different from that of host peptide, or a peptide which showed an increase in labeling after infection. This definition would include any host peptide whose synthesis is induced by phage-

FIG. 2. *Agarose gel analysis of DNA fragments produced by digestion of 1.1 μg of SP82 DNA, 1.7 μg of SPO1 DNA, or 1.4 μg of φe DNA with Hpa II or Hha I. Photograph of an ethidium bromide-stained 0.7% agarose slab gel, with the fragments lettered from top to bottom. DNA fragments produced by digestion with Hpa II: (a) SP82 DNA, (b) SPO1 DNA, and (c) φe DNA. DNA fragments produced by digestion with Hha I: (d) SP82 DNA, (e) SPO1 DNA, and (f) φe DNA.*

regulatory proteins, although it should be noted that UV irradiation was used to decrease host transcription. The assumptions made in this type of analysis have been discussed elsewhere (12).

Overall, the patterns of peptides synthesized by cells infected with SP82 or with SPO1 (Fig. 3) have many similarities and some distinct differences. One of the more striking differences is the apparent synthesis of an intensely labeled band (marked with an arrow in the right margin) in the extracts from SPO1-infected cells very early in infection and the continued synthesis of this peptide throughout the time of sampling; the extracts from SP82-infected and φe-infected cells (Fig. 4 and 5) do not contain this peptide. A second arrow at the bottom of Fig. 3 indicates another SPO1 peptide not produced by SP82-infected cells. Synthesis of this early peptide diminished markedly by the 6 to 9 min interval of labeling. A φe peptide of similar size and interval of synthesis can be seen in Fig. 4. Examination of Fig. 3 yielded an estimate that approximately 46 of the total 70 SP82-specific peptides could be matched with SPO1 peptides with respect to mobilities and intervals of synthesis. Thus, in this one-dimensional comparison, 66% of the peptides produced by SPO1 and SP82-infected cells were identical. The approximate molecular weights of the identical peptides could also be summated and compared with the total molecular weight of all of the SP82 peptides detected. When this was done, a slightly higher value (74%) was obtained ($2.5 \times 10^6/3.4 \times 10^6$).

Similar comparisons of peptides synthesized during infection by SP82 and φe are shown in Fig. 4; Fig. 5 compares peptides produced by SPO1-infected and φe-infected cells. Examination of Fig. 4 gives the estimate that 23 of the 67 peptides (34%) produced by SP82-infected cells were synthesized during the same interval and had identical mobilities with φe-specific peptides. The summated molecular weight of the identical peptides is 44% ($1.4 \times 10^6/3.2 \times 10^6$) of the total molecular weight of the 67 SP82-specific peptides. Two of the more striking examples of peptides specific to φe-infected cells are marked with arrows in the right margins of Figs. 4 and 5; these peptides were not produced by either SP82 or SPO1-infected cells. Examination of Fig. 5 provides the estimate that 23 of the 62 SPO1-specific peptides (or 37%) had identical mobilities and intervals of synthesis with φe peptides. The summated molecular weight of the identical peptides is 47% ($1.4 \times 10^6/3.0 \times 10^6$) of the total molecular weight of the 62 SPO1-specific bands. It should be mentioned that about 10 peptides (or 14%) seem to be identical in all three phage infections. Included in this number is the major phage structural protein of SP82 (12).

In comparing the patterns shown in Fig. 3 to 5, phage peptides having the same mobility and appearing during the same portion of the lytic cycle were considered "identical," regardless of the intensity of labeling; all other peptides were categorized as "different." It is recognized that two-dimensional gel analysis would provide a more reliable assessment of identity and that bands that appear identical in one-dimensional gels can represent different proteins. A single comparison was made by two-dimensional gel electrophoresis (data not shown) of two samples analyzed in the one-dimensional slab gel whose autoradiogram is shown in Fig. 3. This comparison showed that the extract of cells labeled 21 to 24 min after infection with SPO1 contained a total of 112 labeled peptides; the parallel SP82 sample contained 125 labeled peptides. The last two lanes of Fig. 3 had 66 and 56 bands for SPO1 and SP82, respectively. The percentage of identical peptides detected by two-dimensional electrophoresis was 42% (identical peptides/total SP82 peptides = 53/125), and the percentage in the one-dimensional gel was 71% (40/56). Thus, a two-dimensional analysis suggested less similarity between SP82 and SPO1 than analysis by the one-dimensional gel.

Phage neutralization. Neutralization by phage antisera measures only a few specific proteins. Nevertheless, this method has been used for analyzing relationships between phages. Neutralization rate constants (K) were determined as described by Adams (1), using antiserum prepared against SP82. The values for K were 510 for SP82, 240 for SPO1, and 120 for φe. These results cannot be used directly as an estimate of relatedness, but the fact that SPO1 and φe were neutralized by antiserum to SP82 is of interest. It is known that some very closely related phages (e.g., T3 and T7) are neutralized inefficiently by the heterologous antiserum (1).

DISCUSSION

The amount of homology between the genomes of SP82, SPO1, and φe was estimated by DNA-DNA hybridization, by digestion with restriction endonucleases, and indirectly by comparison of phage proteins synthesized during infection. Each of these methods has specific limitations, and quantitation of data derived from analyses with restriction endonucleases and peptide comparisons is difficult. The results (summarized in Table 5) obtained from DNA-DNA hybridization experiments indicated that, at the more stringent conditions, an average of

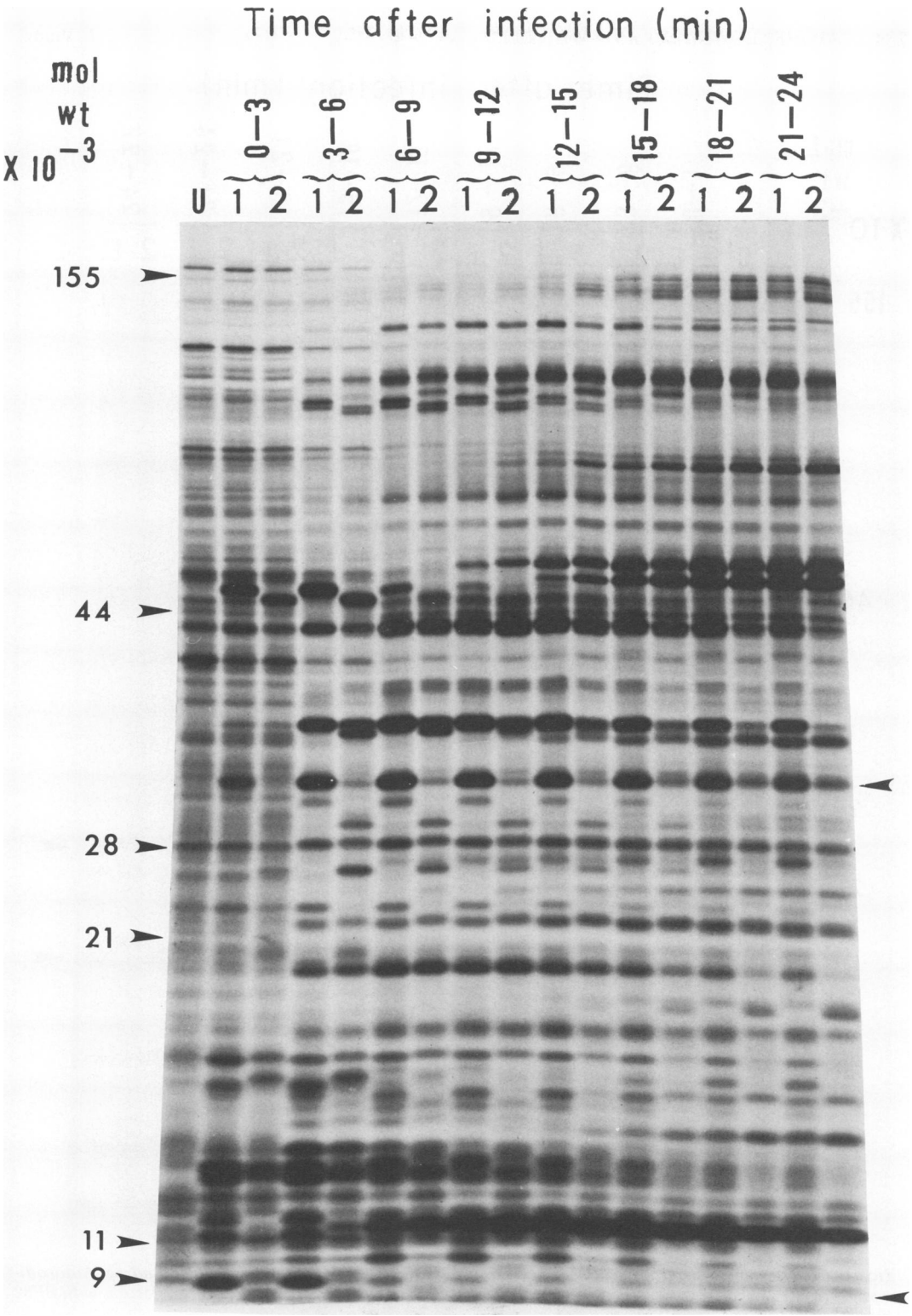

FIG. 3. *Comparison of peptides synthesized during the infection of B. subtilis by SPO1 and SP82. Autoradiograph of a sodium dodecyl sulfate-polyacrylamide slab gel (14 to 20% exponential polyacrylamide gradient) showing uninfected host peptides (U) and peptides synthesized during infection with SPO1 (1) and SP82 (2). The intervals of pulse-labeling with [^{35}S]methionine are indicated. Approximately 100,000 cpm of radioactivity was applied to each well, and the film was exposed for 9 days. Molecular weights were estimated from the mobilities of subunits of RNA polymerase isolated from SP82-infected B. subtilis. The arrows in the right margin indicate peptides described in the text.*

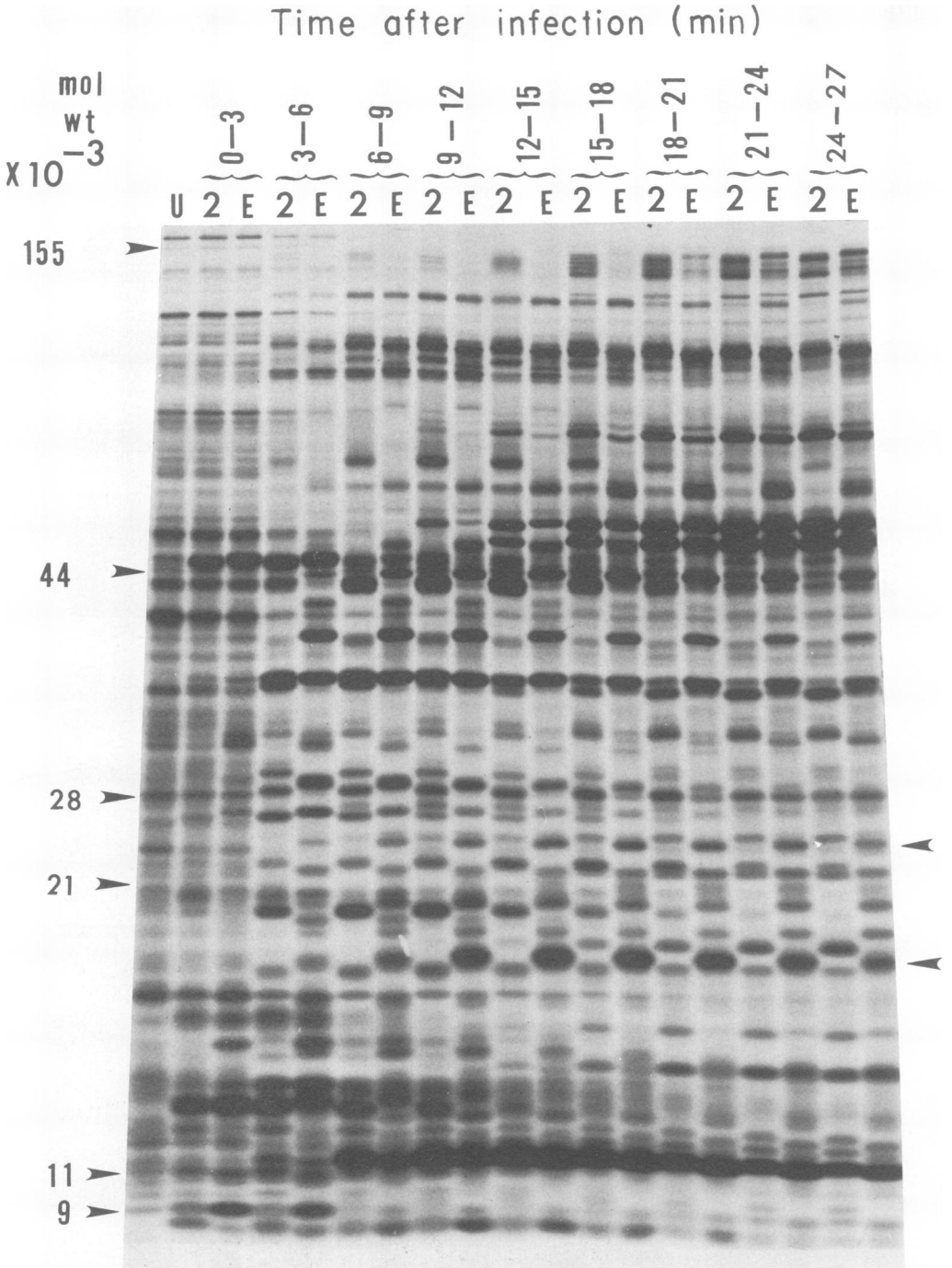

FIG. 4. *Comparison of peptides synthesized during the infection of B. subtilis by SP82 and φe. Autoradiograph as in Fig. 3 showing uninfected host peptides (U) and peptides synthesized during infection with SP82 (2) and φe (E).*

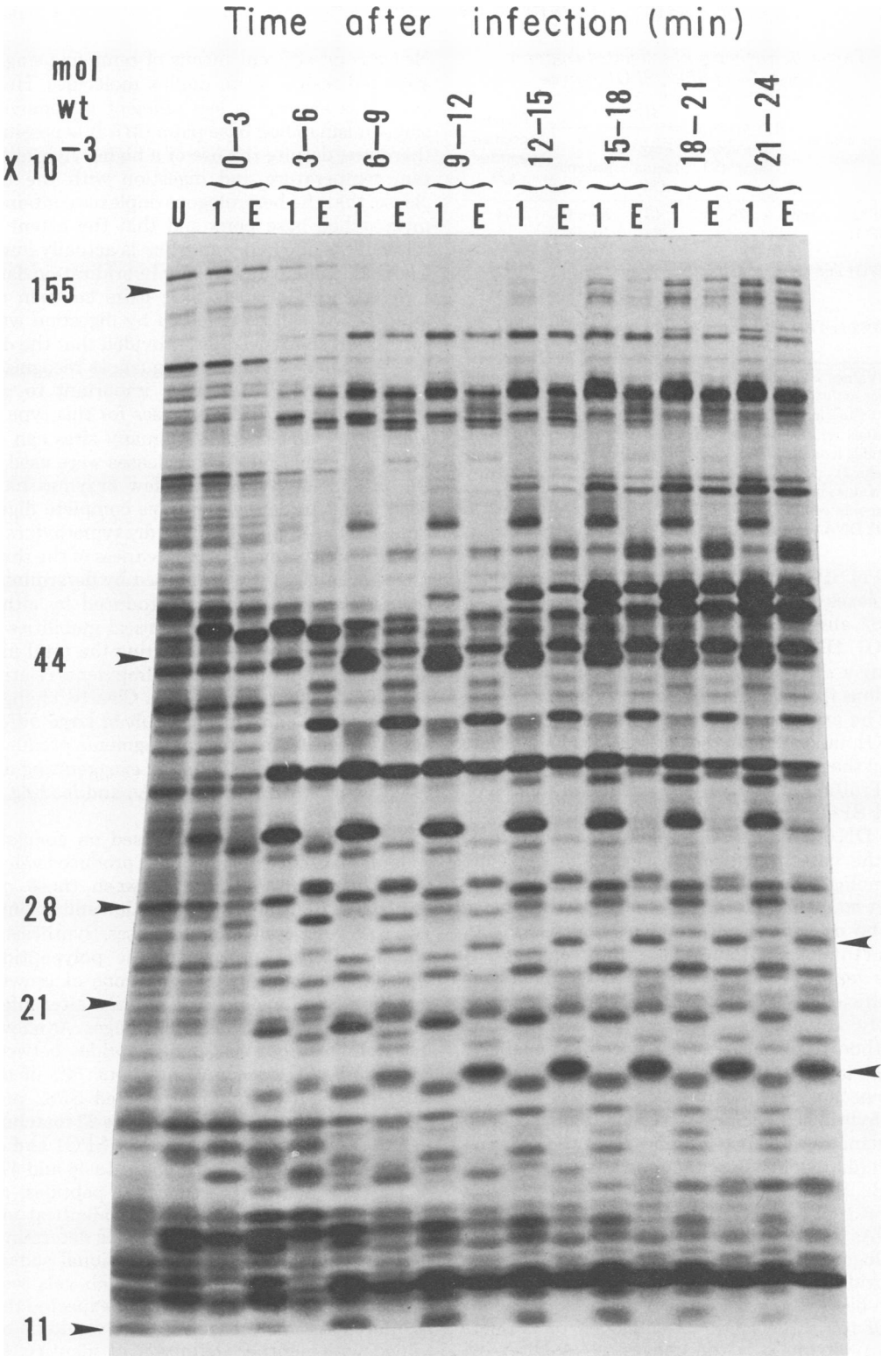

FIG. 5. *Comparison of peptides synthesized during the infection of B. subtilis by SPO1 and φe. Autoradiograph as in Fig. 3, showing uninfected host peptides (U) and peptides synthesized during infection with SPO1 (1) and φe (E). Film exposed for 5 days.*

TABLE 5. *Summary of estimated degree of relatedness of SP82, SPO1, and φe*

Phage comparison	DNA-DNA hybridizations[a] (%)	Restriction		Phage-specified peptides[b] (%)
		Fragments[c] (%)	Enzyme	
1. SP82 and SPO1	78	48	*Hpa* II	74
		64	*Hha* I	
2. SPO1 and φe	41	19	*Hpa* II	47
		8	*Hha* I	
3. SP82 and φe	38	19	*Hpa* II	44
		18	*Hha* I	

[a] Values indicate average of the reciprocal hybridization values at 70°C shown in Table 1.

[b] Values indicate summated molecular weights of matching peptides relative to the total molecular weight of all SP82 peptides (comparisons 1 and 3) or of all SPO1 peptides (comparison 2).

[c] Values indicate summated molecular weights of matching fragments relative to SP82 DNA (comparisons 1 and 3) or SPO1 DNA (comparison 2).

78% of SP82 and SPO1 sequences formed hybrid duplexes, 38% of φe DNA was homologous with SP82, and 41% of φe DNA was homologous with SPO1 DNA. These values were obtained by using a method which removed single-stranded regions from double-stranded duplexes.

The extent of relatedness between SP82 and SPO1 indicated in the present study was less than that suggested by the earlier measurements of Truffaut et al. (25). They reported that SP82 and SPO1 DNAs were 100% homologous with the DNA of phage 2C, implying that all or most of the base sequences in SPO1 and SP82 are homologous. One explanation for this discrepancy could be that the genome of 2C, the labeled probe used by Truffaut et al. (25), is smaller than that of SPO1 and SP82. However, 2C DNA was reported to have the same sedimentation coefficient as SPO1 or SP82 DNA (25). A more likely explanation is that the hybridization method used earlier, i.e., annealing of labeled DNA to filter-bound DNA (4), yielded high values because single-stranded sequences attached to hybridized regions were not removed. This explanation receives support from the observation (data not shown) that annealing the labeled SP82, SPO1, and φe DNAs used in the present hybridization experiments with filter-bound DNAs yielded values of 90 to 100% in the heterologous hybridizations. Similar differences in hybridization values using the two methods have also been reported for bacterial DNAs (20).

Of the three methods employed to evaluate the relatedness of the phages, DNA-DNA hybridization indicated the highest degree of homology. As stated earlier, digestion with S1 nuclease is an efficient means of removing single-stranded regions from duplex molecules. However, this enzyme is less efficient in removing single mismatched base pairs (21). It is possible, therefore, despite the use of a higher hybridization temperature and digestion with the nuclease, that the heterologous duplexes contained mismatched base pairs and that the extent of relatedness of SPO1 and SP82 is actually lower than indicated by the present hybridization data.

Differences in single base pairs between related DNAs can be detected by digestion with restriction endonucleases, provided that the differences alter or generate sequences recognized by the endonucleases. It is important to use many restriction endonucleases for this type of analysis so that changes at many sites can be detected. Only four endonucleases were used in the present study, because few enzymes have been found to date which give complete digestion of DNAs containing hydroxymethyluracil in place of thymine. The relatedness of the three phages (Table 5) was estimated by determining the number of fragments (produced by either *Hpa* II or *Hha* I) having identical mobilities in agarose gels and then calculating the total molecular weight of the matched fragments relative to either SP82 or SPO1 DNA. Clearly, changes in single base pairs might result in large differences in size and number of fragments produced by the endonucleases, thereby exaggerating differences between related DNAs and leading to low estimates of relatedness.

Estimates of relatedness based on comparisons of phage-specified peptides produced values which were intermediate between those obtained by DNA-DNA hybridization and by analysis with restriction endonucleases. Synthesis of approximately 70 phage-specific polypeptides was detected under our conditions of growth, infection, and labeling of *B. subtilis* after infection with any one of the three phages. As shown in Table 5, the 46 matching peptides between SP82 and SPO1 account for about 74% of the total molecular weight of detected SP82 peptides. Similar comparisons with the 23 matching peptides between SP82 and φe or SPO1 and φe show that these peptides amount to 44 and 47% of the detected SP82 and SPO1 peptides, respectively. Only peptides having identical mobilities and intervals of synthesis as determined by electrophoresis on one-dimensional sodium dodecyl sulfate-polyacrylamide slab gels were defined as "matching." It might be expected that a different estimate of relatedness would be obtained with another definition of identity, for example, by using two-dimensional gels. A single comparison did, in fact, indicate a greater diver-

sity between SP82 and SPO1 than was suggested by the one-dimensional electrophoretic analysis. However, it is not certain that a more sensitive method of separating the peptides would provide a better estimate of relatedness, since a change in a single amino acid could alter the mobility without affecting the function of a peptide. It is significant that a large number of peptides seem to be identical by both one- and two-dimensional analysis.

Despite the differences in levels of relatedness estimated from the three types of comparisons, Table 5 shows that the estimates appear internally consistent. All three measurements indicated that SP82 and SPO1 are related to a significantly greater extent than SPO1 and ϕe or SP82 and ϕe. It would be of interest to determine whether data obtained in other comparisons (e.g., genetic maps, studies of complementation, DNA sequencing, electron microscopy of heteroduplexes, transcriptional maps, electrophoretic analysis of phage proteins, etc.) would be consistent with these estimates of relatedness.

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