Bactericidal Activities of Human Polymorphonuclear Leukocyte Proteins against *Escherichia coli* O111:B4 Coated with C5 or C8

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The postnuclear supernatant of disrupted polymorphonuclear leukocytes exhibited bactericidal activity on *Escherichia coli* O111:B4 coated with immunoglobulin M antibodies and C5 or C8 but not on C3- or C7-coated bacteria. To characterize this antimicrobial activity further, granules obtained from the postnuclear supernatant were extracted with sodium acetate (pH 4) and the soluble extract was subsequently fractionated through carboxymethyl cellulose and Sephacryl S-200. Over 90% of the activity present in the starting material was recovered in the soluble granule extract. Kinetic and dose-response analyses of the bactericidal activity of the polymorphonuclear leukocyte extract on BAC1-5 and BAC1-8 revealed different susceptibilities to killing of these two bacterial intermediates; they also differed for their susceptibilities to killing at 37°C and at room temperature. The suggestion raised by these data, that BAC1-5 and BAC1-8 could be killed by different bactericidal factors, was confirmed by the findings that separate fractions of the soluble granule extract obtained by carboxymethyl cellulose and Sephacryl S-200 chromatography exhibited specific activity on either BAC1-5 or BAC1-8, whereas other fractions were active on both intermediates.

The contribution of the complement system to the host defense against bacterial infections is well established and is accomplished essentially through its opsonizing and chemotactic activities, as well as through direct bactericidal effect bon susceptible bacteria. Evidence has also been provided in favor of an additional role of complement in potentiating the bactericidal activity of the phagocytes once the bacteria have been engulfed (6, 8, 10). Thus, components of the alternative pathway promote the intracellular killing of *Staphylococcus* by the monocytes (7) and the late-acting components promote that of *Escherichia coli* O111:B4 by the polymorphonuclear leukocytes (PMN) (15). More detailed investigations performed by our group on the components involved in the processing of *E. coli* O111:B4 by PMN revealed an absolute requirement of membrane-bound C5 or C8 for effective killing of the bacteria (21). These studies also showed that the killing of C5- or C8-coated bacteria could be obtained equally well with the postnuclear supernatant (PNS) of disrupted PMN as with the intact PMN, suggesting that bactericidal factors rather than products of the respiratory burst may be responsible for the increased susceptibility to killing of *E. coli* bearing C5 or C8.

The aim of the present investigation was to characterize the bactericidal factors of PMN active on *E. coli* O111:B4 coated with immunoglobulin M (IgM) and various complement components. Data are presented showing that the killing of bacteria carrying C5 or C8 is promoted by distinct bactericidal factors.

MATERIALS AND METHODS

Buffers. Krebs-Ringer phosphate (KRP) buffer (pH 7.4; ionic strength, 0.091 μ) supplemented with 0.64 mM MgCl$_2$ and 0.16 mM CaCl$_2$ was prepared as previously described (16).

Bacteria and bactericidal assay. The study was performed on the strain of *E. coli* O111:B4 obtained from the stock collection of the Institute of Microbiology, University of Trieste. The bacteria were grown in nutrient broth (Difco Laboratories, Detroit, Mich.) for 6 h at 37°C and then on nutrient agar (Difco) overnight as previously described (16). The standard bactericidal assays were performed by incubating the mixture of 5×10^5 bacteria and either complement components or PMN extract (final volume, 0.2 ml) at 37°C for 30 min. The number of killed bacteria was evaluated by the method of dilution and counting of CFU in nutrient agar. The bacterial killing was expressed as the bactericidal index calculated by the formula 100 − [(CFU with reagent/CFU with KRP) × 100].

Complement components and complement-deficient sera. The following purified complement components, all from human sources, were used: C5 purchased from Calbiochem-Behring, La Jolla, Calif., pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; functionally purified C6 and C7 obtained from Cordis Laboratories, Miami, Fla.; and C8 and C9 purified in our laboratory by the methods of Steckel et al. (19) and Biesecker and Müller-Eberhard (1), respectively. The last two components had only trace contaminants of IgG. The CH50 hemolytic activities of C6 and C7 were those indicated by the manufacturers, and the specific activities of all the other components evaluated on 1.5×10^7 antibody-sensitized erythrocytes for 30 min at 37°C in a total reaction volume of 250 μl (20) were 1,450 U/μg for C5, 1,200 U/μg for C8, and 849 U/μg for C9.

Human sera with selective deficiencies of C5 (14), C6 (22), and C8 (20) were used to prepare the bacterial intermediates (BACs) BAC1-3, BAC1-5, and BAC1-7, respectively. Pooled human sera treated with KSCN and hydrazine hydrate (3) were used as the reagent providing functionally active C6, C7, C8, and C9. The specificity of this reagent was evaluated by its ability to restore the bactericidal activity on IgM-opsonized bacteria (BA) to C6-, C7- (14), and C8-deficient sera and also to kill bacteria coated with complement components up to C8. Conversely, this reagent failed to reconstitute the bactericidal activity to guinea pig C4-
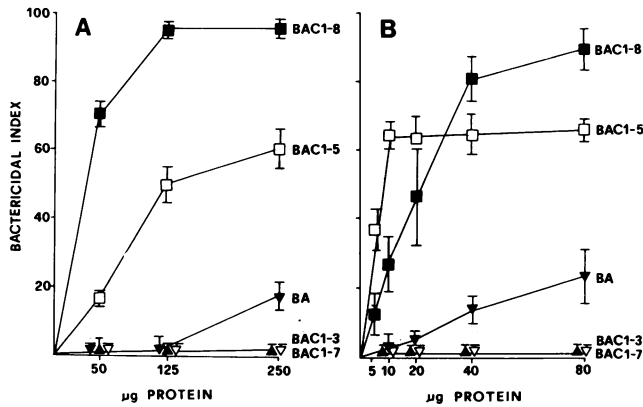
FIG. 1. Bactericidal activity of PNS (A) and of granule acetate extract (B) on BACs. The assay mixture containing 5×10^5 bacteria and various amounts of the PMN extract to a final volume of 0.2 ml in KRP was incubated at 37°C for 30 min. The bactericidal index was evaluated as described in Materials and Methods. Mean values of eight different experiments and standard errors (vertical bars) are represented.

deficient serum (National Institutes of Health, Bethesda, Md.) and human C3- (23) and C5-deficient sera.

Preparation of BACs bearing various complement components. As previously reported (16), BACs were prepared by mixing 3×10^7 BA with optimal dilutions of complement-deficient sera at 0.91 μ ionic strength to a total volume of 300 μl. In particular, BAC1-3 was obtained by incubating BA with the C5-deficient serum at a 1/40 final dilution for 15 min at 37°C, whereas the time required for optimal binding of complement components to bacteria for the preparation of both BAC1-5 and BAC1-7 was 25 min at 37°C by using the C6- and the C8-deficient sera at final dilutions of 1/30 and 1/20, respectively. BAC1-8 was obtained in two ways by incubating either BAC1-7 (3×10^7) with 150 U of C8 or BAC1-5 (3×10^7) with 100 U each of C6 and C7 and 150 U of C8 for 10 min at 37°C. The formation of BACs was revealed by their susceptibility to killing in the standard bactericidal assay after the addition of 15 U of each of the remaining complement components or, when required, of 10 μl of the reagent providing C6 through C9 to 5×10^5 BACs. The percentages of BACs formed under the experimental conditions described above were $\geq 95\%$.

Preparation of the granule extract. The PNS was obtained from homogenized PMN as previously described (21) and centrifuged at $20,000 \times g$ for 30 min at 4°C to prepare a granule-rich pellet. This was suspended at a concentration of approximately 300 mg of proteins, as assayed by the method of Lowry et al. (9), in 100 ml of 0.2 M acetate buffer (pH 4), extracted for 60 min at 4°C with continuous stirring, and finally centrifuged at $40,000 \times g$ for 30 min at 4°C to collect the soluble extract. The procedure was repeated three times and the pool of the three extracts was concentrated by Diaflo ultrafiltration with PM-10 membranes (Amicon Corp., Lexington, Mass.) and dialyzed against 10 mM acetate buffer (pH 5) containing 0.2 M NaCl.

Fractionation of the granule acetate extract by ion-exchange and gel filtration chromatography. As the first step of the fractionation procedure, approximately 150 mg of soluble granule acetate extract was applied to a CM-52 (Whatman, Inc., Maidstone, United Kingdom) column (1 by 6 cm) equilibrated with 10 mM acetate buffer (pH 5) containing 0.2 M NaCl. Fractions of 4 ml were collected at a flow rate of

100 ml/h. The bound proteins were eluted with a two-step linear gradient of increasing NaCl concentration from 0.2 to 0.5 M and subsequently from 0.5 to 1.0 M. The relative salt concentrations of the fractions were determined by measuring the conductivity of the eluates (Radiometer, Copenhagen, Denmark). The active fractions were pooled, concentrated by lyophilization, and applied to a Sephacryl S-200 superfine (Pharmacia, Inc., Uppsala, Sweden) column (1.5 by 120 cm) equilibrated with 10 mM acetate buffer (pH 5) containing 0.7 M NaCl. Fractions of 2 ml were collected at a flow rate of 10 ml/h.

Enzymatic assays. The lysozyme activity was measured by the rate of lysis of *Micrococcus lysodeikticus* estimated as the decrease in turbidity at 460 nm at 25°C by using hen egg lysozyme as the standard. Different volumes of the sample were added to 0.5 ml of the bacterial suspension adjusted to 0.8 to 0.9 optical density (OD) units in 50 mM phosphate buffer (pH 6.6) containing 77 mM NaCl, and the final volume was brought to 1 ml (4). The results were expressed as micrograms of hen egg lysozyme per minute per milliliter of fraction or pool. The myeloperoxidase activity was assayed by the guaiacol method described by Chance and Mahely (2) as modified by Romeo et al. (13). The reaction mixture contained 0.1 M phosphate buffer (pH 7.0), 15 mM guaiacol (Sigma Chemical Co., St. Louis, Mo.), and various volumes of the sample to 1 ml final volume. The reaction was started by adding 0.33 mM H_2O_2 and was followed kinetically at 37°C by measuring the increase in OD at 470 nm (OD_{470}). The results were expressed as micromoles of tetraguaiacol formed per minute per milliliter of fractions or pools. The chymotrypsinlike activity was estimated by the method of Odeberg et al. (12). Benzoyl-L-tyrosine ethyl ester dissolved in dimethyl sulfoxide was used as the synthetic substrate at a concentration of 0.5 mM and mixed with the sample to a final volume of 1 ml by using phosphate-buffered saline (pH 7.4). The hydrolysis of benzoyl-L-tyrosine ethyl ester was monitored spectrophotometrically at OD_{356}, and the results were expressed as the decrease in OD units per minute per milliliter.

RESULTS

Bactericidal activity of PNS and granule acetate extract on BACs. Treatment of various BACs with different concentrations of PNS resulted in a selective killing of BAC1-5 and BAC1-8, whereas both BAC1-3 and BAC1-7 were highly resistant and BA was marginally killed at the highest doses of PNS (Fig. 1A), thus confirming and extending previous observations obtained with PNS (21). The evaluation of the bactericidal activity at each step of the purification procedure of PNS revealed that almost all the bactericidal activity of the starting material was recovered in the $20,000 \times g$ pellet containing the granules and over 90% of this activity was eventually recovered in the soluble acetate extract of the pellet. Essentially, the soluble extract gave a pattern of activity similar to that of PNS on the various BACs, except for a higher specific activity (Fig. 1B). BAC1-8 proved to be equally susceptible to similar amounts of PMN extract, whether it was prepared from BAC1-7 and purified C8 or obtained from BAC1-5 and purified C6, C7, and C8. In addition, both preparations of BAC1-8 showed an increased susceptibility to killing by the crude PMN extract compared with that of BAC1-5. Although BAC1-5 and BAC1-8 were both found to be susceptible to the bactericidal activity of PNS and granule acetate extract, some differences were noticed in the behavior of the two BACs. The killing of

BAC1-8 was dose dependent and reached values of over 90% at the highest concentration of both PNS and granule extract. In contrast, the average percentage of killed BAC1-5 never exceeded 65%, which in the case of the acetate extract was obtained with 10 μg, and was not changed appreciably by a further increase in the protein concentration. The two BACs also showed some differences in their susceptibilities to killing when the time and temperature of incubation were varied (Fig. 2). In fact, the killing of BAC1-5 was complete within 10 min and was equally efficient both at 37°C and at room temperature, whereas BAC1-8 was killed only at 37°C and in any case more slowly than BAC1-5. The kinetics of BA killing were essentially similar at the two temperatures, but the extent of killing was markedly low.

Effect of granule acetate extract on the stability of BAC1-5. One possibility which could explain the incomplete killing of BAC1-5 by the granule acetate extract was that enzymes present in the extract itself might favor the decay of BAC1-5 by stripping the bound complement components off the bacterial membranes. This hypothesis was tested by incubating BAC1-5 for 30 min at 37°C with the lowest dose of the acetate extract which caused maximal killing of this intermediate. At various intervals, samples were withdrawn and assayed for the percentage of BAC1-5 killed by the acetate extract and for the number of surviving bacteria still in this intermediate state, as measured by their susceptibility to killing after addition of the residual late-acting complement components. BAC1-5 suspended in KRP served as the control for the spontaneous decay of this BAC. The results (Fig. 3) show that BAC1-5 mixed with the acetate extract had only a slight decay of about 20%, similar to that of BAC1-5 in KRP at the end of the 30-min incubation. However, BAC1-5 was stable at 10 min, when maximal killing by the acetate extract occurred.

Fractionation of the bactericidal activity from the soluble acetate extract. The different susceptibilities to killing of BAC1-5 and BAC1-8 raised the question as to whether distinct bactericidal factors were responsible for the selective killing of each of the BACs. This question was ad-
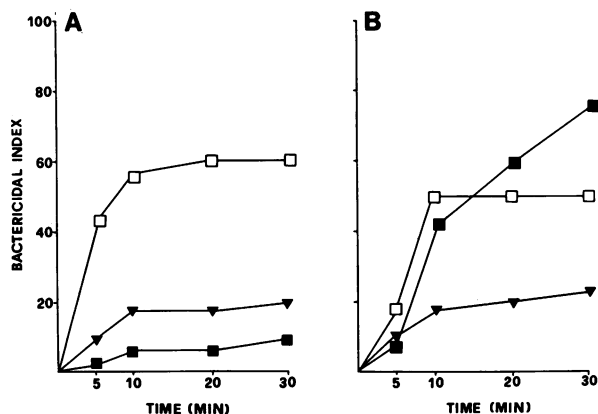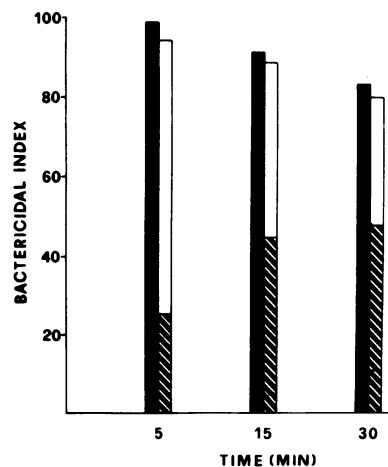

FIG. 3. Effect of granule acetate extract on the stability of BAC1-5. The bacteria were mixed with the granule acetate extract as described in the legend to Fig. 2 and incubated for up to 30 min at 37°C. At various intervals, two samples of 25 μl were removed, diluted 1/50 with cold KRP, and incubated for 10 min, either in KRP (▨) to measure the number of bacteria killed by the acetate extract or with human reagent providing the complement components from C6 to C9 (□) to evaluate the number of surviving bacteria still in the state of BAC1-5. As the control for the spontaneous decay of BAC1-5, the bacteria were incubated with KRP (■) and mixed at various intervals with the remaining components of the complement sequence for further 10 min.

dressed by fractionating the soluble acetate extract by CM-52 and testing the bactericidal activity of the fractions on BAC1-5 and BAC1-8. The same fractions were also assayed on BA as the control for nonspecific activity. The chromatographic profile (Fig. 4) shows three distinct groups of fractions acting either on both BAC1-5 and BAC1-8 (pools 1 and 3), which elute at conductivities from 28 to 39 mS and from 53 to 69 mS, respectively, or selectively on BAC1-8 (pool 2), eluting at conductivities from 40 to 47 mS. None of these pools was active on BA, but some activity on BA was detected in the exclusion peak. Assays of the pools for known cationic proteins of PMN showed that pool 1 contained about 100 and 80% of the recovered lysozyme and myeloperoxidase activities, respectively, whereas all the recovered chymotrypsinlike activity was present in pool 3. The residual 20% myeloperoxidase activity was detected in pool 2. The unabsorbed proteins eluted with the starting buffer did not show any bactericidal activity. When further fractionated by a Sephacryl S-200 column, pools 1 and 3 resolved in several peaks acting on BAC1-5, BAC1-8, or both (Fig. 5). In particular, fractions of pool 1 active on BAC1-5 eluted late, together with a major peak of proteins, whereas the bactericidal activity for BAC1-8 was distributed in two distinct peaks. Pool 3 had a totally different elution pattern, with an early peak of bactericidal activity on BAC1-5 and two additional peaks acting both on BAC1-5 and BAC1-8.

DISCUSSION

A variety of potent bactericidal proteins differing in molecular weights and electrophoretic mobilities were isolated from the granules of PMN of different species (5, 11, 17, 24, 26, 27). These proteins often show selective activities on various bacterial species, which include both gram-positive

FIG. 2. Kinetics of bactericidal activity of granule acetate extract on BA (▼), BAC1-5, (□), and BAC1-8 (■), at room temperature (A) and at 37°C (B). A 0.2-ml mixture containing 5 × 10⁵ cells of BA, BAC1-5, or BAC1-8 and the minimal amounts of granule extract giving the maximal bactericidal activity (10 μg for BAC1-5 and 40 μg for BA and BAC1-8) was incubated for 30 min at either 37°C or room temperature. At various intervals, 25-μl samples were removed, diluted 1/50 with cold buffer to block any further bactericidal activity of the PMN extract, and assayed for the number of killed bacteria.

and gram-negative bacteria (18). The results of our study demonstrate the presence in the granule-rich fraction of additional bactericidal activities for *E. coli* O111:B4 only when it is coated with IgM antibodies and C5 or C8. This strain of *E. coli* was shown to be relatively resistant to killing by the PMN cationic proteins compared with its rough mutants (11, 25), and this has been attributed to the long polysaccharide chain of lipopolysaccharide (25). This relative resistance is maintained after the binding of the IgM-specific antibodies to the outer membranes. Interestingly, the assembly of the complement components at various steps of the complement sequence on IgM-coated *E. coli* O111:B4 can variably modify the susceptibility of these bacteria to killing by both PNS and granule acetate extract. Thus, the binding of C5 or C8 renders the bacteria susceptible to killing, whereas the coating of the membrane with the early components up to C3 does not affect their resistance. In addition and for reasons not yet clear, the susceptible BAC1-5 can again be transformed into a resistant intermediate after the assembly on the bacterial membrane of the trimolecular complex $\overline{C567}$ which leads to the formation of BAC1-7.

There are several reasons to believe that distinct bactericidal factors and probably different mechanisms are responsible for the killing of BAC1-5 and BAC1-8. First, the time and temperature of incubation differently affect the survival

FIG. 5. Sephacryl S-200 gel filtration of pools 1 (A) and 3 (B) obtained from CM-52 chromatography of granule acetate extract. The chromatographic profiles are depicted in the top half of the figure. In the bottom half of the figure, the bactericidal activities of the pooled fractions (15 μl) on BAC1-5 (■) or BAC1-8 (⊠) are represented. The protein contents of the various pools in the 15-μl samples were as follows: (A) 1, 7 μg; 2, 3.1 μg; 3, 45 μg; (B) 1, 1.2 μg; 2, 1.3 μg; 3, 2.9 μg.

of the two BACs. BAC1-5 was killed within 10 min both at 37°C and at room temperature, whereas BAC1-8 was only killed at 37°C, reaching the maximum at 30 min of incubation. Second, the killing of BAC1-8 was on the whole more extensive than that of BAC1-5, particularly at the maximal concentrations of PNS or granule extract. The reason 30 to 35% of BAC1-5 escaped killing by PNS or acetate extract could be explained neither by an intrinsic decay of BAC1-5 nor by an extrinsic decay induced by the soluble acetate extract. In fact, we previously showed that this BAC is rather stable (16), and the results presented in Fig. 3 indicate that its stability is not affected by incubation with the granule extract. The finding that over 90% of BAC1-5 could be killed by some fraction pools of Sephacryl S-200 gel filtration rather suggests the presence of interfering substances as yet undefined in the more crude preparations of PNS or acetate extract. Further supporting evidence for distinct bactericidal factors acting selectively on BAC1-5 or BAC1-8 is provided by the isolation of separate chromatographic fractions specific for each of the two BACs. The observation that more than one fraction is active on the same target is not unusual since similar findings were reported by other groups (5, 11, 17). The relationship of any of these factors with the already described bactericidal cationic proteins is unknown, and work is in progress to characterize further the molecular structure of these proteins and their mechanism of action. The in vivo role of the membrane-bound late-acting complement components in the processing of gram-negative bacteria by PMN bactericidal proteins both in the body fluids and in the circulation remains to be established.
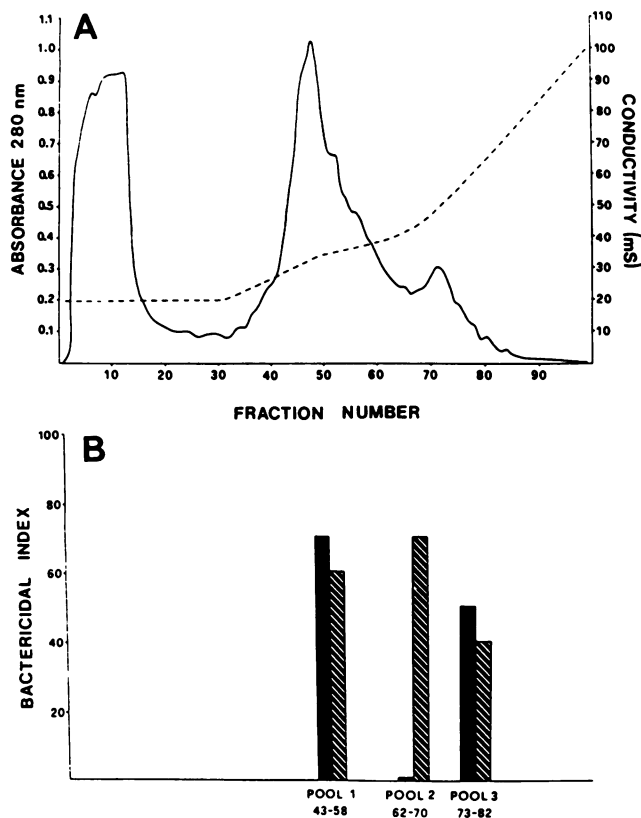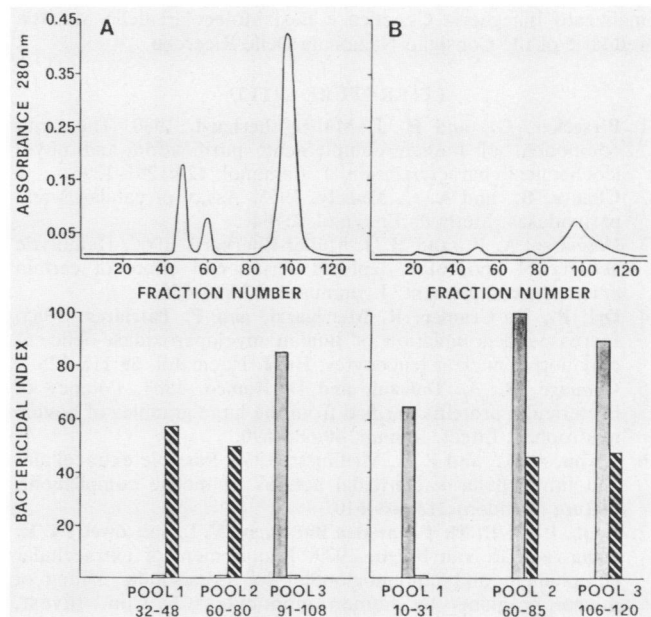

FIG. 4. (A) Fractionation of the granule acetate extract by CM-52-cellulose by using a two-step linear gradient. About 150 mg of protein were applied to a CM-52 column (1 by 6 cm) equilibrated with 10 mM acetate buffer (pH 5) containing 0.2 M NaCl. The first gradient from 0.2 to 0.5 M NaCl was applied at fraction 30, and the second gradient from 0.5 to 1.0 M NaCl was started at fraction 68. (B) The bactericidal activities of the pooled fractions (30 μl) on BAC1-5 (■) or BAC1-8 (⊠).

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