Bacteria of the order Actinomycetales are one of the most important sources of bioactive natural products, which are the source of many drugs. However, many of them still lack efficient genome editing methods, some strains even cannot be manipulated at all. This restricts systematic metabolic engineering approaches for boosting known and discovering novel natural products. In order to facilitate the genome editing for actinomycetes, we developed a CRISPR-Cas9 toolkit with high efficiency for actinomycetes genome editing. This basic toolkit includes a software for spacer (sgRNA) identification, a system for in-frame gene/gene cluster knockout, a system for gene loss-of-function study, a system for generating a random size deletion library, and a system for gene knockdown. For the latter, a uracil-specific excision reagent (USER) cloning technology was adapted to simplify the CRISPR vector construction process. The application of this toolkit was successfully demonstrated by perturbation of genomes of Streptomyces coelicolor A3(2) and Streptomyces collinus Tü 365. The CRISPR-Cas9 toolkit and related protocol described here can be widely used for metabolic engineering of actinomycetes.